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Treating spinal cord injury with a combination of human fetal neural stem cells and hydrogel modified with serotonin agonists.

Léčba míšního poranění za pomoci kombinace lidských fetálních neurálních kmenových buněk a hydrogelu modifikovaného serotoninovými agonisty.

Master of Science thesis

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Affirmation

I hereby declare that I have written this Master of Science thesis independently, with the use of the listed literature and my own research.

Prague 27.4.2011 Jiří Růžička

Signature

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Abstract

Spinal cord injury (SCI) results in the loss of nervous tissue and consequently the loss of motor and sensory function. The transplantation of neural stem cells (NSCs) and a porous hydrogel material may support spinal cord repair. In my Master of Science thesis we evaluate the biocompatibility of the human fetal neural stem cell (hfNSC) line SPC-01_GFP3 in combination with hydroxy ethyl methacrylate hydrogel modified with a serotonin agonist (P2544-1). Moreover, we evaluate the effect of a combination of SPC-01-derived progenitors and P2544-1 hydrogel on functional improvement and tissue reconstruction. As a model of SCI, a spinal cord lateral hemisection at the Th8-9 level in adult Wistar rats was used. A P2544-1 hydrogel seeded with SPC-01 cells was applied immediately after the hemisection surgery (n=11) in the treated animals, while the control group was only hemisected (n=20). Locomotor (BBB) and sensitivity (plantar test) evaluations were performed weekly for three months. An immunohistochemical analysis (IHC) of the cells and hydrogel was made *in vitro* before the surgery and also at the conclusion of the experiment. IHC and the behavioural tests showed that this combination of NSCs and hydrogel material is highly biocompatible *in vitro*, but that after transplantation it was unable to quickly stimulate the ingrowth of endogenous nervous and capillary system elements and that the cells that persisted in the hydrogel survived only in low numbers. A major portion of the transplanted cells successfully migrated and proliferated out of the lesion, but the only positive effect on the surrounding tissue was decreased astrogliosis. The treatment did not lead to functional improvement, except for short-term stabilisation of the nerve circuits and increased survival of treated animals. Only the sensory test revealed a functional trend of increased thermal sensitivity compared to the controls. In general, the treatment of SCI in a hemisection model by a combination of hfNSCs seeded on a P2544-1 hydrogel led to limited functional improvement within the time constraints of the experiment. These results were possibly influenced by the inadequacy of the hemisection model itself. On the other hand, the human fetal neural stem cell line SPC-01 appears to be promising for cell therapy thanks to its migratory, survival and neural phenotype potential. In combination with a hydrogel to enable more convenient *in vivo* transplantation, the use of these cells may possibly lead to significant improvement in functional outcome.

Key words: SPC-01, neural stems cells, hydrogel, spinal cord injury, hemisection, hydrogel surface modification,

Abstrakt

Míšní léze je příčinou poškození nervové tkáně vedoucí ke ztrátě lokomočních a senzorických funkcí. Aplikace neurálních kmenových buněk v kombinaci s porézním hydrogelem přemosťujícím lézi je jednou z cest, která může podpořit regeneraci míchy. Cílem mé práce bylo zjistit biokompatibilitu specifické kombinace lidských fetálních neurálních kmenových buněk (hfNSCs) SPC-01 linie a hydroxy-etyl-metakrylatového hydrogelu s navázanými molekulami serotoninového agonisty (P2544-1), a její efekt na rekonstrukci poškozené tkáně a celkové funkční zlepšení. *In vivo* část pokusu byla prováděna na modelu laterální hemisekce míchy (n=31). Léčené skupině byl následně po hemisekci implantován hydrogel porostlý buňkami SPC-01 linie (n=11) a obě skupiny byly farmakologicky ošetřeny (kontrola n=20). Po následující dobu 12 týdnů byly prováděny lokomoční a senzorické testy pro vyhodnocení funkčního zlepšení. Před aplikací a po ukončení pokusu byly hydrogely s kmenovými buňkami imunohistochemicky (IHC) analyzovány. Z celkových IHC a behaviorálních analýz pak vyplývá, že kombinace neurálních kmenových buněk SPC-01 linie a P2544-1 hydrogelu je vysoce biokompatibilní *in vitro*. V agresivním prostředí poškozené míchy však hydrogelový materiál atrahuje jen část potřebné endogenní tkáně, čímž pravděpodobně není zajištěn dostatečný tok informací a metabolitů. Jen malá část aplikovaných buněk přežívá v prostředí hydrogelu, zatímco většina migruje z prostředí léze. Aplikované buňky si zachovávají neurální fenotyp. Statisticky významný vliv této kombinace na rekonstrukci okolního prostředí byl prokázán pouze u snížení zajizvení okolní tkáně. Vliv kombinace SPC-01 buněk a P2544-1 hydrogelu na celkové lokomoční a senzorické zlepšení je omezen na počáteční protekci zachovaných axonálních spojů. Tento efekt však v průběhu studie přestává být signifikantní a zůstává pouze nesignifikantní trend ve zvýšené citlivosti k tepelnému podnětu u léčené skupiny. Léčba míšního poranění za pomoci kombinace neurálních kmenových buněk SPC-01 linie a hydroxy-etyl-metakrylatového hydrogelu s navázanými molekulami serotoninového agonisty v rámci modelu hemisekce míchy potkana přináší jen zlepšení daného poškození funkčně se neprojevující a celkové zvýšení imunitní odolnosti. Lidské fetální neurální kmenové buňky SPC-01 linie se ovšem prokázaly být slibným zdrojem pro buněčné terapie a v kombinaci s vhodnějším materiálem by mohly dosáhnout signifikantního funkčního zlepšení v modelech míšního poranění.

Klíčová slova: SPC-01, neurální kmenové buňky, hydrogel, míšní léze, hemisekce, povrchové modifikace hydrogelu

Contents

Index of abbreviations

AA- Amino acid

AMPAR-2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl) propanoic acid receptor

ASIA- American Spinal Injury Association

bbb- Blood brain barrier

BBB- Basso, Beattie, Bresnahan locomotor test

BDNF- brain-derived neurotrophic factor

BMSC- Bone marrow stromal cells

cAMP- cyclic adenosine mom phosphate

CNS- Central nervous system

CSPG- Chondroitin sulphate proteoglycan

DMEM- Dulbecco's modified Eagle's medium

DRG- Dorsal root ganglion

ECM- Extracellular matrix

EGF- Epidermal growth factor

ESC- Embryonic stem cell

FACS- Fluorescence activated cell sorter

FGF- Fibroblast growth factor

GCSF- Granulocyte-colony stimulating factor

GDNF- Glial-derived neurotrophic factor

GFAP- Glial fibrillary acidic protein

GFP- Green fluorescence protein

GRP- Glial restricted progenitors

HE- Haematoxylin-Eosin staining

HEMA- Hydroxy ethyl methacrylate

HPMA- Hydroxy propyl methacrylate

ChABC- Chondroitinase ABC

IHC- Immunohistochemical staining

IKVAV- Isoleucine-lysine-valine-alanine-valine- primary AA sequence of laminin binding site

iPS- induced pluripotent stem cells

IVC-Internally ventilated cages

MA- Methacrylate

MACS- magnetic activated cell sorter

MAG- Myelin-associated glycoprotein

MOETACl- [2-(methacryloyloxy)ethyl] trimethylammonium chloride

MPSS- Methyl prednisolone sodium succinate

MSC- Mesenchymal stem cell

NASCIS- National spinal cord injury studies

NCAM- Neuronal-cell adhesive molecule

NCSC- Neural crest stem cell

NF70- Neurofilaments 70

NF160- Neurofilaments 160

NGF- Neuronal growth factor

NT3- Neurotrophin 3

NT4/5- Neurotrophin 4/5

NSC (hfNSC)- Neural stem cells (human fetal neural stem cells)

OEG- Olfactory ensheathing glia

OPC- Oligodendrocyte precursor cell

PBS- Phosphate-buffered saline

PCR- Polymerase chain reaction

PEG- Polyethylene glycol

PNN- Perineuronal nets?

P2544-1- HEMA hydrogel with covalently anchored serotonin agonist molecules

RECA- anti-endothelial cell antibody

RGD- Arginine- glycine- aspartic acid- primary AA sequence of fibronectin binding site

SCI- Spinal cord injury

SGZ- Subgranular zone

Shh- Sonic hedgehog

SPC-01- specific hfNSC cell line derived from 8-week-old fetal spinal cord

SVZ- Subventricular zone

TNF-α- Tumor necrosis factor- α

UCBSC- Umbilical cord blood stem cell

VEGF- Vascular endothelial growth factor

Wnt- wingless/int-1

YIGSR- Tyrosine- isoleucine- glycine-serine-arginine- part of primary AA sequence of laminin

4-OHT- 4 hydroxy tamoxifen

1. Introduction

SCI is one of the most common traumatic injuries with lifelong consequences. No successful treatment has been developed yet. After the primary insult, unselective massive cell death and ongoing secondary processes render the microenvironment unsuitable for endogenous regeneration. Current surgical techniques and pharmacotherapy help to stabilize the environment but are unable to restore lost function or even prevent ongoing side effects. Long term research has shown that combined treatment may affect more processes of the secondary injury, providing a better chance for functional improvement. For example, the release of pharmacologically active molecules or the mimicking of healthy endogenous tissue by hydrogel materials in the area of the lesion have been shown to be useful approaches to accelerate endogenous repair mechanisms. The use of hydrogels that partially imitate the endogenous tissue and that serve as a bridge for the penetration of endogenous cell has become a question of various combinations of modifiable features. In this way, hydrogel materials are not only passive non cellular bridges, but can also serve as a source of an attractant gradient. Thanks to the application of different types of stem cells, damaged or dead cells can be replaced, and thanks to the paracrinal stimulation of the used stem cells, implantation helps to reconstruct the lesioned tissue. The optimal combined treatment of spinal cord injury with modified bridging hydrogel materials and specific neural progenitors or stem cells can help tissue reconstruction, which is necessary for the acceleration of regeneration. These combinations will hopefully lead to partial or complete functional improvement.

2. Theoretical overview

2.1. Models and clinical classification of spinal cord injury

The principle division of SCI is primarily based on the absolute damage value, the injury location (cranio-caudal relation, Fig.1.), the cause of the injury and the length of time that has passed from the injury.

Based on the degree of damage, SCI cases are divided into complete and incomplete (severe and mild) injuries. In clinical practice the damage is graded according to the American Spinal Injury Association (ASIA) impairment scale (Tab.1.), or the Frankel score, ranging

from “A” with no observed movement or sensory response below the damaged spinal level to “E”, which means a healthy subject (Tab.1) (Ditunno et al, 1994; Reier J., 2004).

The injuries can also be divided into three groups defined by the extent of paralysis: paraplegia, tetraplegia and pentaplegia. Complete and incomplete paraplegia represents 41,5% of all cases and tetraplegia 52,4% (Lim et al., 2007).

The third classification of injury is associated with the primary cause. There are two groups of injuries: open injuries caused by the penetration of sharp objects (laceration), and compression closed injuries represented by maceration, contusion, massive compression and solid cord injury (Hulsebosch C., 2002; Thuret et al., 2006).

In animal models the open injuries are represented by hemisection and complete transection. Closed models of injury most often include the spinal clip model, the weight drop model and a balloon compression lesion (Kakulas, 1987). In clinical settings, compression injuries are more frequent compared to open injuries. A contusion injury represents 25 to 40% of SCI cases (M. Oudega et al., 2005). Both open and closed models of SCI are used in research. A model of compression injury, such as a balloon compression lesion, can provide more information about possible clinical application. Compared to the weight drop model and the spinal clip model, the epidural compression model creates a lesion a few segments above the entrance into the spinal cord and eliminates the influence of a laminectomy on SCI treatment. The model of compression injury mimics the clinical situation of patients with a spinal cord injury more closely compared to sharp models (transection, hemisection). The effect of treatment in models of closed injuries is best demonstrated using behavioural testing methods, as opposed to the open injury model of hemisection. However, compression models of SCI are more demanding in terms of postoperative care and bring a danger of urinary tract infections (Vanicky et al., 2001). Open injury models of hemisection, on the other hand, show the penetration of bridging materials (using IHC) more clearly, because of the sharp border between the spinal cord and the lesion or graft (Hejcl et al, 2009; Wei et al, 2010).

The fourth very important system of classifying SCI is related to the length of time that has passed since the injury. From the clinical point of view, injuries are categorized as acute, sub-acute or chronic. Acute injury occurs within a few days from the primary cause of damage. The subacute phase occurs within a few days to a few weeks after the original damage. The chronic phase is in the range of months to years.

A combination of these four factors – the location of the lesion, the absolute damage value, the type of damage and the time factor – defines the treatment model. In an acute injury model, an implant is inserted immediately after the injury, whereas in sub-acute and chronic

models of SCI, the implantation of a bridging graft is performed following a delay of up to five weeks post-injury. Differences in the suitable time window for the implantation of a stem cell-seeded hydrogel exist between open and closed models of SCI. Glial scar resection, which is necessary for implanting the bridging graft, more influences closed injuries and presents a possible clinical problem in chronic injury treatment. In the hemisection model, the glial scar resection has only a small or no influence on the process of treatment (Rasouli et al., 2009). In clinical settings the approach of acute implantation is not feasible. However, a certain delay between the insult and the implantation of hydrogel materials can have a positive effect on recovery/regeneration/repair. In experiments reported by A. Hejcl, the subacute application of a seeded hydrogel resulted in a decrease in the size of cavity formation in the surrounding damaged tissue compared to acute injury treatment. Both treatment time windows, acute and subacute, showed positive results compared to the control group. These results indirectly point to a reduction of the secondary damage of the spinal cord (Hejcl et al., 2008 A). The different reactions of opened and closed injury models to the surgery described above raises the question as to whether treatment approaches utilizing biomaterial implantation can be sufficiently effective to overcome the additional damage caused by the resection of the glial scar (Rasouli et al., 2009).

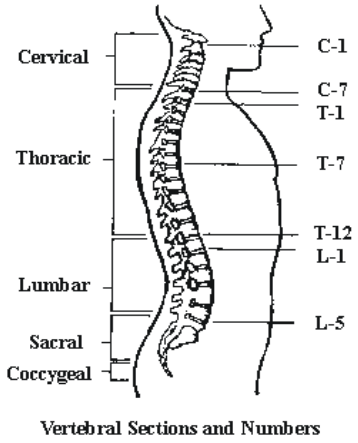


Fig.1. Anatomical nomenclature of cranio-caudal direction of spinal cord (www.ASIA.com).

Tab.1. American Spinal Injury Association scale

ASIA GRADE	Functional deficit	description
A	**Complete**	No motor or sensory function in the lowest sacral segment (S4-S5)
B	**Incomplete**	Sensory function below neurologic level and in S4-S5, no motor function below neurologic level
C	**Incomplete**	Motor function is preserved below neurologic level and more than half of the key muscle groups below neurologic level have a muscle grade less than 3.
D	**Incomplete**	Motor function is preserved below neurologic level and at least half of the key muscle groups below neurologic level have a muscle grade 3.
E	**Normal**	Sensory and motor function is normal

Legend: Five grades of SCI clinical evaluation visualised in the ASIA scale. Evaluation of each muscle yields/results in five degrees of preserved function (www.ASIA.com).

2.2. Behavioural evaluation

For the evaluation of proper recovery, it is necessary to identify a suitable combination of behavioural tests. Today, a wide variety of behavioural tests that are directed towards specific nervous circuits or that evaluate complete locomotor behaviour is available.

The outcome of a behavioural test and the animal's behaviour during the procedure are related not only to the extent of the damage, but also to the injury model. In experiments in the Metz laboratory, two hundred animals with two different types of lesion – dorsal hemisection and contusion injury – were used. The results showed a different relationship among a series of behavioural tests depending on the type of injury and on the Basso, Beattie, Bresnahan (BBB) test score (tab.2). With respect to this connectivity, the authors defined a priority for using tests that complement the BBB test outcome. Animals with BBB scores lower than 13 (tab.3) were more connected to the open field activity test (second priority test). Grid walking and kinematics tests were classified as third and fourth priority tests. Animals with a score higher than 13 on the BBB test scale were more capable in grid walking and narrow beam tests (second priority test), and foot print analysis together with kinematics tests were classified as third and fourth priority tests. Each type of injury causes a different type of damage to the spine with its own processes, and any circuits can be less or more preserved (Metz et al., 2000).

The most common test combination is the use of the BBB locomotor test (Basso et al., 1996) together with sensory tests, such as a hot plate test for heat nociception (Montagne-Clavel and Oliveras, 1996) and/or the von Frey filaments test for measuring tactile allodynia (Takaishi et al., 1996). Despite its subjective component, the BBB test is the fundamental locomotor test, and in almost all injury models it represents the first choice test for quantifying recovery. One important problem has been observed with the use of the BBB test. The main stimulus for the animal to move is the fear of an open field, which is not always a strong enough impulse and thus the animals do not feel the need to increase their movements. An additional impulse for moving implies using an additional locomotor test capable of increasing the attractiveness of movement. One of the locomotor tests that forces animals to engage in locomotor activity and also serves as a clinical rehabilitation procedure is treadmill training. The advantage of treadmill training in its positive influence on locomotor recovery and weight bearing in cats and humans was not confirmed in rats with an incomplete lesion (Fouad et al., 2000). Another advantage of the treadmill test is the possibility of simultaneously utilizing electromyography (EMG) (Li et al., 2011) or 3D movement analysis

(Canu and Garnier, 2009). In recovery, the process of training also plays an important role in promoting neural plasticity and enhancing regeneration (Girgis et al, 2007).

In order to collect objective data, not influenced by the observer or by specific aspects of the testing procedure, it is necessary to maintain the same conditions throughout the whole experiment. Based on experience with behavioural testing methods, the conditions necessary for a reliable outcome have been defined.

Five basic rules have been derived for obtaining complete and uninfluenced results: First is providing the animals with adequate nutrition. The second rule is to ensure freedom from injury and disease complications (by using antibiotics and safe housing). The third important rule is to prevent post-surgical thermal and physical discomfort. The fourth principle is the opportunity to express normal patterns of behaviour. The last important rule is to keep the animals out of stressful conditions that prevent the animals from performing the required task (Tatlisumak and Fisher, 2006). In light of research into animals' daily rhythms, another condition for objective data collection has to be fulfilled. Rats are crepuscular and nocturnal animals, and the observer has to optimize the testing procedure for the most convenient circadian time (subjective animal day time) and maintain it regularly in all testing sessions. The optimal time in the case of rats is in the morning or evening hours (Kriegsfeld et al., 1999).

For some behavioural procedures, pre-training of animals is necessary to teach them how to perform the task and to habituate them to the testing conditions. During the training procedure, a healthy animal´s score is elevated, and this score can serve in the experiment as a positive control. The frequency of testing procedures after surgery is dependent on the time that has elapsed from the operation and the test's specificity. The question is when is testing immediately after surgery actually necessary and when can a short time delay preserve post-injury damage (Sedy et al., 2008).

Via a careful choice of behavioural tests and animal care procedures, the results obtained will describe not only the health of the animal, but also the progress of the injury and both positive and negative effects of the treatment. Such information is important for the preclinical evaluation of defined SCI treatments.

Tab.3. Behavioural test priorities for objective results

Type of the injury	First choice test	Second choice test	Third choice test
Cervical	Forelimb asymmetry	Footprint analysis	BBB
Th. compression	BBB	Hot plate	Inclined plane
Th. contusion	BBB	Electrophysiology	Von Frey/hot plate
Th. hemisection	BBB	Electrophysiology	Von Frey/hot plate
Th. transection	BBB	Electrophysiology	Kinematics analysis
Th. excyttoxic	Hot plate	Cold testing	Von Frey
Th. ischemic	BBB	Electrophysiology	Inclined plane/hot plate
Other injury	BBB	Electrophysiology	Hot plate/ gird walk

Legend: Recommendations concerning the use of behavioural testing methods for different types of SCI based on an analysis of studies published during the past 12 years (Sedy et al., 2008).

2.3. Regeneration after spinal cord injury

Currently, there is no evidence of a complete treatment for spinal cord injury. Even if in acute spinal cord injury some motor and sensory improvements are possible, chronic injury is still open for innovative methods, in spite of the low endogenous regeneration potential (Hejcl et al., 2010).

2.3.1. Primary damage

Primary damage consists of the direct or indirect responses to the mechanical force or load. The primary insult is caused by a variety of loading conditions including flexion, extension, axial load, rotation, or distraction. The extent of damage is connected to the type of load. Under a dynamic load, a small force with a short stimulus to attenuate the CNS insult is necessary. On the other hand, under a static load, the spinal cord reacts so as to save itself from greater damage, until the force overloads its natural flexibility. The response is characterised by nonspecific cell loss as well as sublethal damage. In spite of that, a cascade of secondary responses leading to prolonged cell death, network dysfunction, and system level changes are activated (LaPlaca et al, 2007).

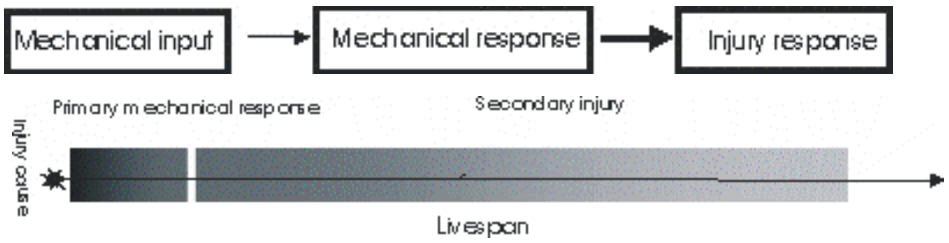


Fig. 2. SCI biomechanics (LaPlaca et al., 2007)

2.3.2. Secondary damage

The secondary damage is influenced by the primary insult, as well as the health and the age of the individual. The rupture of cells and the primary tissue damage initiate secondary injury mechanisms. Mechanical disruption of the vasculature results in petechial haemorrhage and intravascular thrombosis. This leads, together with vasospasms of intact vessels and edema of the injured tissue, to hypoperfusion and ischemia. Due to oxidative stress and the mechanical distortion of membranes, extracellular abnormalities together with ionic shifts occur (Kwon et al., 2004; Young and Koreh, 1986). The uncontrolled increase of glutamate concentration activates extrasynaptic N-methyldiaspartate receptors. This extrasynaptic activation has an opposite effect on neuronal survival compared to a synaptic signal. Clc1 is one of the activated pathways leading to neuronal destruction (Wahl et al., 2009).

These processes continue during the secondary injury. After reperfusion of the blood, high levels of oxygen induce the peroxidation of surface and cell components. Oxidized radicals are induced due to changes in the myelin sheaths. This leads, together with overloaded dematurated astrocytes, to partial demyelination and astrogliosis (Fitch and Silver, 2008). After disrupting the blood brain barrier (bbb), the resulting damage and edema can activate the immune system. The problem is that the immune system, which is normally restricted behind the bbb, has both positive and negative impacts, and in response to injury the immune system causes massive cell death. It has been shown that under normal conditions, tumour necrosis factor-α is responsible for the incorporation of the GluR2 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid receptors (AMPARs) in the process of memory encoding and learning. In the lesion environment with its high glutamate concentration, this process of AMPAR amplification leads to excytotoxicity (Ferguson et al., 2008). The influence of a number of chemokines on secondary injury processes has been

demonstrated. Blocking their receptors has both a positive and negative impact on injury treatment (Gal et al., 2009).

Both apoptotic and necrotic cell death are observed after SCI. The extrinsic and intrinsic apoptotic cascades have been closely examined to find convenient targets for the treatment of secondary damage and approaches for the convenient application of anti-apoptotic factors have been examined (Lee et al., 2009; Vanderhaeghen et al.,2010). These secondary processes continue for days to months, and the loss of signal guidance to the injured neurones involves retrograde and anterograde axonal degeneration. The demyelination of nerve fibres continues. A newly formed astrogliotic barrier created by astrocytes, oligodendrocytes and ependymal cells overexpressing Perineuronal nets (PNN) and extracellular matrix (ECM) subunits enclose the lesion and the surrounding pseudocysts and cavities. Those processes mentioned previously make the lesion microenvironment unfavourable for endogenous regeneration. Reconnection of newly formed axons often leads to malfunctioning and allodynia. A small number of spared axons nearly always persist. In the beginning the treated animals are often hyperalgesic (Yaksh et al., 1999), but the preservation of spared axons by means of suppressing secondary processes leads in turn to the preservation of their physiological function. Even a small number of spared axons can preserve important physiological functions, and so protecting the tissue from secondary injury damage makes a difference in the quality of life (Blesch et al., 2002).

2.3.3. Endogenous regeneration

Research from the last decade has shown that in the adult central nervous system (CNS), the major part of neuronal proliferation and differentiation persists in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus. Neurogenesis in the adult brain led to the idea of using endogenous NSCs for CNS repair. However, the autologous transplantation of NSCs into different parts of the CNS revealed the role of the local environment in guiding stem cell differentiation and such transplantation was not sufficient for larger lesions. Except in the hippocampal environment, NSCs generated glial cells rather than neurones (Ortega-Perez, Murray et al., 2007; Cao et al., 2002). Because of the influence of the environment, differentiation directed towards the neural phenotype, as observed in the SVZ and the SGZ, is not apparent in spinal cord injury experiments. Nonetheless, NSC implantation into the spinal cord induces gliogenesis, which is also necessary for the reconstruction of lesioned tissue (Ronaghi et al., 2009; Kumagai et al., 2009; Walczak et al., 2011).

2.3.4. Clinical intervention and research approaches

The pharmacological potential of SCI research promises a range of new treatment methods compared to those in current clinical use. Clinical interventions include surgery and the use of high-dose steroids, followed by neurorehabilitation using such approaches as treadmills and body-weight support training.

Lesion decompression at the right time window alleviates secondary damage (Fehlings et al., 2010). However, decompression surgery is not the final step, and another intervention to stabilise or support the lesion environment is still necessary.

One of the interventions that are used to stabilise the lesion environment is pharmacological therapy. Pharmacological interventions for acute SCI treatment include corticosteroids, antibiotics and/or gangliosids as well as antagonists or inhibitors of intrinsic ion channels and receptors (Kwon et al., 2004). One of the first choice corticosteroids is methylprednisolon in the form of derivate methylprednisolon sodium succinate (MPSS). In the National Spinal Cord Injury Studies (NASCIS), the active doses and time window for the application of MPSS were defined. In these same studies, opioid receptor antagonists, calcium channel blockers and lazaroids were compared with MPSS. The opioid receptor antagonist naloxone, tirilazad mesylate (a member of the 21-aminosteroid family of antioxidant "lazaroids" with inhibitory effects on lipid peroxidation similar to those of MPSS without glucocorticoid side effects) and nimodipine (a calcium channel blockers) were compared in different studies to MPSS treatment or in were used in combined treatment together with MPSS. However, none of them had a more significant effect on motor or sensory system recovery than MPSS. The reason why naloxone, tirilazad mesylate and nimodipine displayed no additional effect has been explained in different studies as the result of applying a subtherapeutic dose. The effect of naloxone treatment was observable only on motor and sensory recovery in patients with incomplete injury (Kwon et al., 2004).

Although MPSS treatment has a limited effect on sensory and motor recovery after SCI, the glucocorticoid side effects of high doses of MPSS have led to a search for safer drug application. One of the alternatives to MPSS treatment are U-74006F and YM-14673 steroid equivalents. Many attempts have been made to solve the problem of the increased positive effects resulting from high doses on the one hand and the higher risk of mortality due to infection pathways on the other, via combination treatment with calcium channel blockers or tetracycline-like antibiotics. The application of nimodipine partly suppressed the negative effect of MPSS treatment, but had no additional effect on motorsensory recovery (Behrmann

et al., 1994; Pointillart et al., 2000). Both effects, the suppression of secondary infection and a positive influence on spinal cord injury regeneration, have been provided by the tetracycline-like antibiotic minocycline (Saganova et al., 2008).

Even the detailed mechanism of MPSS action is not fully understood; currently, MPSS is the only pharmacotherapy used in acute injury treatment. The main issue for clinical and pharmaceutical intervention consists of the high cost and limited effectiveness of the therapy procedures. Together with the basic MPSS treatment, antibiotics and rehabilitation procedures, many other supporting and stabilizing drugs are necessary. The lifetime cost of treatment of a tetraplegia patient from the age of 25 has been estimated in the year 2006 to be $ 2.9 million USD (Lim et al., 2007). However, only a small improvement and stabilisation of the SCI thanks to surgery and pharmacological therapy can be achieved. Even if future pharmacotherapy (involving chemokines, glutamate receptor and ion channel antagonists, etc.) is developed, a more comphersive approach including a combination of bridging the lesion, cell replacement therapies and reconstructing the lesion environment with pharmacotherapy is necessary to achieve complete SCI treatment (Blesch et al., 2002; Jones et al., 2001; Sykova et al., 2006).

2.3.5. Role of extracellular matrix (ECM) compounds in SCI

One of the most important roles in astrogliosis is played by the ECM. Under physiological conditions, molecules of the ECM, particularly their sequences of primary amino acids that are necessary for cell contact information, are responsible for cell growth, cell survival and proliferation. However, after injury those same molecules represent a barrier to cell growth and axonal reconnection. The most important role in the process of astrogliosis is played by ECM proteins such as myelin-associated molecules and chondroitin sulphate proteoglycans (CSPGs). Under normal conditions these molecules, such as neurocan, brevican, phosphacan, agrecan and other CSPGs, together with tenascin-R (one of the myelin-associated molecules) and cartilage link protein-1(ctrl1), serve as elements of the PNNs (Fig.3) between astrocytes and neurones in the grey matter as well as in the white matter, although with different composition (Fawcett and

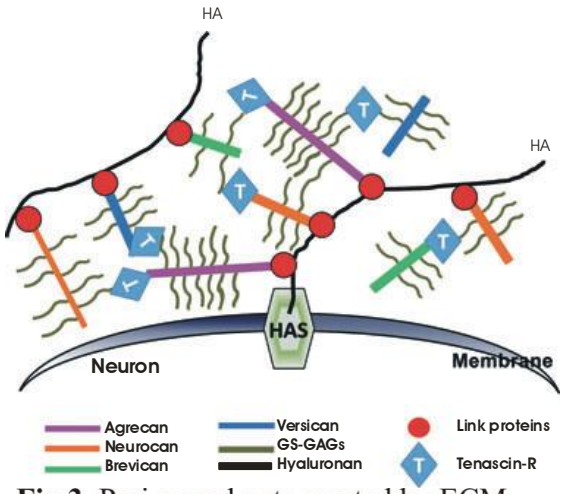


Fig.3. Peri neural nets created by ECM molecules and link proteins (Kwok et al.,2010).

Asher., 1999; Kwok et al., 2010). These link proteins and surface molecules are partially involved during the migration of neural crest cells and in guiding and directing axons (tab.3).

In the postnatal ECM CSPGs are not so tightly bound and do not create a network, therefore the neural environment is more permissive for new axonal sprouting (Kwok et al., 2008). One of the roles of the ECM in an adult organism is keeping the system stable by the additional mechanical restriction of axonal oversprouting. As a consequence of the overexpression of myelin-associated molecules and CSPGs after CNS trauma, mechanical and chemical barriers that disturb axonal sprouting, molecule signalling and metabolite clearance are created (Gervasi et al., 2008). To overcome these barriers, it was shown (Kwok et al., 2008; Barritt et al., 2006) that the bacterial enzyme chondroitinase ABC (ChABC) can be used as a soluble factor to disintegrate PNNs. The disintegration of PNNs increases neural plasticity and plays an important role in the combined treatment of SCI. The route of ChABC administration for effective PNN degradation has been shown to be very important. Thermo-stabilisation or encapsulation of ChABC in a hydrogel environment helps to more effectively degrade PNNs due to the longer sustained delivery of an effective dose of chondroitinase ABC, which in turn has a positive effect on the sprouting of new axons through the lesion. The encapsulation of ChABC is about 37% more effective in reducing glycosaminoglycans 3 weeks after injury compared to the direct administration of ChABC: 24% of the original ChABC molecules were detected following encapsulation, compared to only 4% of ChABC molecules following direct injection. (Lee et al., 2010 A; Hyatt et al., 2010)

Myelin-associated glycoprotein (MAG) and NI250/nogo, overexpressed in damaged myelin regions, are other myelin-associated molecules that are responsible for blocked regeneration after spinal cord injury. NI250/nogo has a subtype called nogo A. Nogo-A is mostly expressed on the surface of myelin and acts on the post-injury environment through a calcium-dependent mechanism (Fawcett and Asher, 1999). However, the primary roles of nogo-A and MAG in the organism were poorly understood for a long time. It has been shown that one of their roles is in the wrapping of axons by oligodendrocytes. In the late development of the CNS, the combined deletion of both nogo-A and MAG causes hypomyelination. However, the deletion of only one of them has no observable effect on myelin sheaths or the nodes of Ranvier (Pernet et al., 2008). In early development nogo-A acts not only on oligodendrocyte precursor cells, but also on neurons. At the present time, it is clear that the role of nogo-A is connected to neuronal cell adhesion molecules (NCAMs) and guidance factors such as ephrin and semaphorin. Due to this connection with guidance receptors and molecules, Nogo-A is involved in developmental brain cortex defasciculation of

nerve fibres due to the negative regulation down-regulation of axon-axon adhesion, growth inhibition and support for neurite branching (Petrinovic et al., 2010). The treatment of nogo-A and MAG over-expression is promising. For example, when using anti-nogo-A antibody or a bridging material binding nogo 66 receptor antagonists, the suppression of astrogliosis has been observed. In addition, a modified hyaluronic acid hydrogel served as an attractive environment for the infiltration of gap43-positive neurons, a marker of axonal sprouting (Wei et al., 2010).

2.3.6. Neuronal growth/ trophic factors and guidance molecules

It is generally known that the presence of trophic factors has an important role in neural development in terms of the differentiation and guidance of migrating glial cells, neurones and their axons (Marler et al., 2010; Riaz et al., 2002). In lesioned spinal tissue, where the lesion microenvironment decreases the regeneration potential of the nervous system, the presence of trophic and growth factors is not only helpful, but even necessary. A suitable combination of trophic factors released from implanted materials and/or cells results in enhanced regeneration (Jones et al., 2001; Willerth et al., 2007).

In the adult neural system, many growth factor families and axon guidance factors are present. The majority of early and late morphogenic factors control cell proliferation as well as the early differentiation (Wingless (Wnt), Sonic hedgehoc (Shh), nodal etc.) and terminal differentiation (fibroblast growth factor (FGF), epidermal growth factor (EGF)) of neural precursors. However, in an adult organism they also act as guidance molecules. On the other hand, axon guidance molecules together with receptors are not only attractive or repulsive factors, but also can serve as neuromodulators or second messengers.

The families of trophic factors that have shown an intrinsic functional outcome in CNS regeneration by exerting a direct effect on neurons and glial cells include the neurotrophin family, the transforming growth factor-β family and the neuregulin family (produced by Schwann cells in peripheral nervous system) (Carroll et al., 1997; Jones et al., 2001). The neurotrophin family consists of four proteins - nerve growth factor (NGF), neurotrophin 3 (NT-3), neurotrophin 4/5 (NT- 4/5) and brain-derived neurotrophic factor (BDNF). These molecules, together with glial-derived neurotrophic factor (GDNF) from the TGF-β family, are the most frequently used trophic factors to support neural regeneration (Blesch et al., 2002).

Even if they represent only a small part of the trophic and differentiation factors that contribute to the process of injury regeneration, they are often used because they act directly

on neurons and glial cells. Other factors that are necessary or helpful in lesion reconstruction include vascular endothelial growth factor (VEGF), factors modulating the immune system such as granulocyte colony stimulating factor (GCSF) or Flt3, and tropic factors such as FGF and EGF. VEGF more likely has a positive effect due to vascularisation and endogenous cell stimulation and could be effectively used as a cotreatment factor (Kim et al., 2009). A combination of more factors can lead to a different or increased response compared to the application of each of them individually. The combination of G-CSF together with Flt3 ligand increases the regeneration of the lesioned environment due to the activation of CD11-positive cells, the facilitation of axonal sprouting and a reduction of astrogliosis in a balloon compression model of acute SCI in the rat (Urdzikova et al., 2011)

Through a different set of receptors, neurotrophic factors influence axonal pruning, proliferation signals and apoptosis. Whereas NT-3 activates the trkC receptor present on axons and can therefore induce cortico-spinal growth, BDNF trkB receptors are closer to the neuronal soma and therefore their primary function is to prevent apoptosis after axonal damage (Blesch et al., 2002).

Somewhere between a trophic factor and guidance molecule is 3´-5´-cyclic adenosine monophosphate (cAMP). cAMP molecules serve internally as second messengers and activators of many signalling pathways between primary receptors and effector enzymes. Less known is its function via Epac receptors, whereby activation of cAMP intracellular level induces robust axonal regeneration or axon attraction/ repulsion (Peace and Shewan, 2011).

Combinations of axonal guidance molecules such as ephrin, semaphorins and nethrins and their receptors from the neurophilin, plexin and eph families are responsible for the axonal attraction and repulsion system. As has been mentioned before, the role of these molecules is ambivalent, and they are often connected with proneurotrophins (Marler et al., 2010). A role for them in cortical structure development has been observed. Molecules of the ephrin, semaphorin, nethrin and other guidance molecule families, together with their receptor, also have a role in synaptogenesis and apoptosis (Feldheim and O´Leary, 2010; Vanderhaeghen and Cheng, 2010).

2.3.7. Cell-based therapy and bridging materials

The need for more effective and safer therapies has led researchers to search for other approaches useable in the acute therapeutic window. Cell-based therapies have been shown to be an additional source of cells for replacement and for support of endogenous cell

regeneration. In addition, the implanted cells can serve as a source of trophic factors that prevent secondary damage (Nandoe Tewarieet al., 2009).

The stem cell approach seems to be promising because of the ability of stem cells to generate cells with a neural phenotype, both *in vitro* and *in vivo*. Embryonic stem cells can differentiate into each germ layer cell precursors and finally, they can even create not only terminally differentiated tissue, but also adult stem cells, such as NSCs. Adult stem cells are mostly capable of differentiation into restricted cell lineages within one germ layer and their proliferative ability is decreased. Different sources of stem cells have been tested to create neural cell populations. To avoid the risk of tumor or teratoma formation after the implantation of pluripotent stem cells, better characterisation and *in vitro* predifferentiation are necessary. For safety and a better understanding of neural development, hESCs and NSCs were cultured *in vitro* and differentiated into a neural phenotype. The process of ESC differentiation into neurones and glial cells is characterised by the use of different methods, including fluorescence activated cell sorting (FACS), magnetic activated cell sorting (MACS), polymerase chain reaction (PCR), and immunohistochemical (IHC) staining (Cai and Grabel., 2007; Kozubenko et al., 2009; Riaz et al., 2002).

Nowadays, there are many stem cell types used, such as hESCs, neural crest stem cells (NCSCs), fetal neural stem cells (fNSCs), adult neural stem cells (NSCs), mesenchymal stem cells/bone marrow stem cells (MSCs/BMSCs), umbilical cord blood stem cells (UCBSCs) and induced pluripotent stem (iPS) cells. (For more detailed information about stem cells and their developmental pathways, see chapter 2.4.).

All these cell types are promising tools for bridging cysts or cavities, replacing dead cells and stimulating endogenous neurogenesis and the remyelination of denuded axons. However, there are large differences between them not only in terms of their proliferation, expansion and differentiation capabilities, but also in their migratory ability and the factors that they produce (Cai and Grabel., 2007; Delfino-Machin et al., 2007; Hu et al., 2010; Karimi-Abdolrezaee et al., 2006).

The injured spinal cord is a poor microenvironment for cell survival, differentiation and maturation. The application of stem/progenitor cells alone, without any modification of the lesion environment, will lead only to partial recovery after SCI. The genetic modification of stem cells and/or their combination with other supporting cells or materials that can simulate the CNS microenvironment is needed for the successful survival of transplanted cells (Thuret et al., 2006; Williams and Lavik, 2009). For combining with stem cells, supporting cells such as Schwann cells, olfactory ensheathing glia (OEGs), or activated macrophages can

be used (Blesch et al., 2002). Schwann cells and OEGs (glial elements from the PNS and olfactory bulb or mucosa) are capable of stimulating fibber regrowth as well as remyelinating denuded fibres themselves (Reier, 2004). Schwann cells have been shown to be capable of forming a supportive cell bridge through the lesion, which can also serve as an active feeder layer for transplanted stem cells. Since Schwann cells also produce their own trophic factors from the neuregulin family, they can be, together with OEGs and ChABC, an alternative approach for SCI regeneration (Oudega et al., 2005).

Nonetheless, even if cell combinations are capable of surviving for a longer time in the lesion, the implementation of tissue engineering principles to promote further tissue repair and regeneration is necessary. Tissue engineering is based on the combination of cells, growth factors and artificial materials with similar properties as the endogenous microenvironment. Polymer scaffolds can be used not only for mechanical bridging of the lesion gap, but also were shown to be important for mimicking molecules of the ECM and/or PNN (Rosso et al., 2005). To mimick the natural environment suitable for cell growth, porosity, structure and chemical composition that resemble the CNS environment have to be utilized.

These materials can be arranged in several groups with respect to their origin, composition, degradability, growth support, and axon guidance ability. Of course, they have to satisfy the basic criteria for biological implants. First, they must be immunologically inert. Second, their physical properties should be similar to the CNS ECM environment, and the material should be capable of trophic factor diffusion. Further, the biomaterial must be able to serve as a supporting structure for spinal cord tissue regeneration (axons, glial cells, blood vessels...). The graft should be soft enough not to damage the surrounding tissue during the process of implantation, fully adhere to the spinal stumps and completely fill the cavity. If the material is resorbable then this process must not create nontoxic metabolites (Hoffman A., 2002; Ramakrishna et al., 2001) (Tab.4.).

Based on their origin, these scaffolds are divided into natural materials, based on ECM elements such as collagen, fibrin or hyaluronic acid, or synthetic materials; structurally, these synthetic materials not only resemble the natural environment, but they are also simple to prepare and can be degradable as well as non-degradable. A special class of synthetic materials are semisynthetic/ composite materials. These materials are composed of different compartments with different properties. Each compartment can therefore be filled and/or covered with a different substrate (growth factors, cells, molecules of the ECM etc). They can be prepared either as degradable or stable materials. The internal structure of these materials depends on the type of polymerization and monomer subunits. Their bridging ability is

enhanced by directed channels or oriented fibres (Novikov et al., 2002; Novikova et al., 2003). Therefore, polymers resembling the natural environment have become a potential solution for the regeneration of various forms of tissue damage. Polymers mimick the ECM, and their combination together with stem cells and soluble or bound trophic factors leads to positive results in spinal cord regeneration (Lee et al., 2010 B).

At the early research stages, these bridging materials were designed with no porous orientation, and therefore they created a chaotic network of channels. To create directed cell growth, either nanofibres or hydrogels with oriented pores can be employed. In the case of nanofibres materials, the direction of cell growth is dependent on the nanofibres' width and orientation (Tysseling-Mattiace et al., 2008). For hydrogels, the design of axonal guidance is more complicated. On one side are porous tubes (Reynolds et al., 2008) and on the other side hydrogels with pores created by the type of polymerisation (porogens, inter-subunit space etc.; Yu et al., 2005). To keep the pores stable throughout the entire length of the hydrogel, some changes in material rigidity are required, which can negatively affect the mechanical properties of the material and therefore the materials do not smoothly adhere to the spinal cord tissue.

Tab.4. Features of biocompatible materials.

Factors	Description		
1st Level material Properties	Chemical/ Biological properties Chemical composition (Bulk and surface)	Physical characteristic Density	Mechanical/ structural characteristic Elastic modulus Poisson´s ratio Yield strength Tensile strenght Compressive strenght
2nd level material properties	Adhesion	Surface topology texture, roughness)	Hardness Shear modulud Shear strenght Flexular modulus Flexular strenght
Specific functional requirements (Based on application)	Biofunctionality(non-trombhogenic, cel adhesion, tec.) Bioinert (non-toxic, non-irritant, non-alergic, non-cancerogenic, etc.) Bioactive Biostability (rsistant to corosion, hydrolysis Oxidation, etc.) Biodegradation	Form(solid, porous,coating film, fiber, mesh, powder) Geometry Coefficient of thermal expansion Electrical conductivity Color, aessthetics Refractivi index Opacity or translucency	Stifness or rigidity Fracture toughtness Fatigue strenght Creep resistence Friction and wear resistence Adhesion strenght Impact strenght Proof stress Abrasion resistance
Processing and Fabrication	Reproducibility, quality, sterizability, packaging secondary procesability		
Characteristic of host:	Tissue, organ, species, age, sex,race, health condition,activity, systemic response, Medical/surgical procedure, period of application/ usage, cost		

Legend: Description of the capabilities and characteristics of artificial biomaterials necessary for effective treatment (Ramakrishna et al., 2001).

To date, complete infiltration of the bridging material with all types of endogenous tissue elements has not yet been achieved (Geller et al., 2002). One of the possible solutions might be to create in the bridging material several compartments containing high levels of tropic factors, which can be continuously released into the environment. This approach will maintain trophic factors or guidance molecules at a stable effective level for a long time (Brandl et al., 2009 Lee et al., 2010 B) (for more detailed information see chapter 2.5.).

2.3.8. Combination strategies

In recent years it has become evident that combination strategies will play an important role in SCI treatment and that a multimodal approach will have a better chance to lead to functional improvement and tissue regeneration. When used in combination with biomaterials and/or trophic factors, transplanted stem cells, have a better chance to survive in the hostile lesion environment (Lee et al., 2010 B).

Advances in molecular biology bring not only new targets based on blocking chemokine receptors (Gal et al., 2009) or apoptotic pathways (Lee et al., 2009), but also reprogramming stem cells or creating iPS cells (Hu et al., 2010) and genetically modified neural precursor cell lines. These immortalized cell lines can be easily expanded *in vitro* and after transplantation, these cells survive and differentiate under *in vivo* conditions better than primary cultures of NSCs (Pollock et al., 2006).

Different current approaches use a combination of related or unrelated factors, cell therapies and/or artificial tissue materials. The artificial materials can be filled with trophic molecules slowly released into the surrounding damaged tissue. The main goal of combination therapies is to improve axonal ingrowth, establish a connection between the implant and the host tissue and reduce glial scarring surrounding the lesion. For example, the combination of BMSCs/ NSCs together with G-CSF accelerates neurogenesis and microglial activation *in vivo*. GCSF acts as a mitotic factor, which stimulates the proliferation of grafted cells (Pan et al., 2008; Yoon et al., 2007). Another interesting and promising approach is the transplantation of neural precursors or MSCS seeded on hydrogels with covalently bound growth factors or primary amino acid (AA) sequences of the ECM. These combinations can positively affect even the chronic injury environment (Aizawa et al., 2008; Hejcl et al., 2010; Woerly et al., 2001).

Strategies using several factors require more complex experiments. The combination of NSCs, endothelial cells and a degradable hydrogel in M. F. Rauch's experiments increased blood vessel density in the hemisectioned spinal cord and helped to create the blood brain

barrier *de novo*. The combination led to a fourfold increase in functional vessels compared to animals with a lesion only, implanted with a hydrogel only, or implanted with NPCs only, and a twofold increase in functional vessels compared to animals that received implants with endothelial cells alone (Rauch et al., 2009). The results from experiments using a combination of material and cell precursors *in vitro* often differ from the *in vivo* situation, where the surrounding tissue presents a different environment compared to *in vitro* conditions. Thus, *in vivo* animal models are always necessary for testing these complex strategies.

2.4. Stem cells and neuronal precursors

Stem cells are defined as cells that have the capacity to self-renew, clonality and the ability to generate differentiated cells. The self-renewal ability of stem cells means that such cells are capable of extensive proliferation without oncogenic transformation. The proliferative capacity of different stem cell types correlates with their pluripotency (Tab.5). The clonality of stem cells is the capacity of a single cell to create more stem cells with identical markers and capable of following the same differentiation pathways. This feature is fundamental for the system of self-renewal and for the creation of a homogenous cell population. A key difference exists among stem cells in their ability to generate differentiated cells. The stem cells that can generate an entire organism are termed totipotent; an example of such cells are germ cells. The stem cells that can differentiate into cells of the three different germ layers (ectoderm, mesoderm and endoderm) are called pluripotent. Multipotent stem cells are capable of giving rise to multiple cell types of only one of the germ layers. Unipotent stem cells can differentiate into a restricted type of cell and have limited proliferative capacity. This type of cell is probably best described as progenitor cells.

Tab.5. The proliferative capacity of several of the most frequently used types of human stem cells (Chai and Leong, 2007)

Type of stem cells	Average doubling time
Embryonic stem cells	35h
Hematopoietic stem cells	45 weeks
Mesenchymal stem cells	1,3-16 days
Neural stem cells	4 days
Embryonic germ cells	3,2 days

2.4.1. Embryonic stem cells

Embryonic stem cells (ESCs) were one of the most exciting topics in stem cell biology during the last decade of the 20th century as they have the capacity to contribute to all somatic tissues. Their pluripotency makes them an abundant source of cells for replacement therapy on the one hand, but a somewhat risky tool on the other hand. The transplantation of undifferentiated ESCs into immune-deficient animals implicitly leads to teratoma formation (Kondo et al., 2007). Therefore, the development of well-defined differentiation protocols as well as an intensive analysis of the derived precursor and progenitor cells are extremely

important for both clinical application and also experimental use. Despite the clonality of stem cells, the population of derived progenitors can be very heterogeneous, and immunofluorescence methods, such as a fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS) (Cai and Grabel., 2007; Ronaghi et al., 2010), have to be used to enrich the desired population. Another important criteria that has to be monitored throughout an experiment or clinical trial with ESC-derivates is cariotype and genetic stability.

2.4.2. Markers of pluripotency and differentiation pathways

Undifferentiated hESCs are characterised by the expression of the pluripotent markers Sox2, Oct3/4, nanog, SSEA-4 and TRA-1-60. During differentiation into a neural phenotype, the expression of these pluripotent markers is decreasing and neural-specific markers are appearing. However, not every population of cells cytometricaly characterised as neural precursors is safe and can be used for transplantation. HESC-derived neural precursors with an expression marker profile of nanog ^{low}/ SSEA-4 ^{low}/ TRA-1-60 ^{low}/ NCAM ^{high}/NF70 ^{high}/ βIII-tubulin ^{high}/ Nestin ^{high} resulted in tumour formation in 50% of cases after transplantation into the rat brain. Long term propagation in vitro and further FACS analysis showed that the low expression of CD15 (SSEA1) and CD24 and the high expression of CD133 are very important for successful transplantation, whereas the high expression of HLA-ABC and the low expression of CD271 (NGFR) result in the decreased viability and migration of cells after transplantation (Kozubenko et al., 2010). In addition to these markers of neuronal stem cell precursors, Sox2, Sox3, Oct2 and Pax6 participate in ESC differentiation and characterise the anterior neuroectoderm (Cai and Grabel, 2007).

ESC differentiation protocols in vitro usually imitate endogenous developmental pathways. Whereas Wnt, Shh, bone morphogenic protein-4 and nodal play important roles at the beginning of ESC differentiation, EGF, FGF together with neurotrophic factors are important at later stages. The combination of Wnt/β-catenin, Nodal and Shh signalling promotes neuronal differentiation in stages from embryonic bodies to neural stem/progenitor cells (NS/PC). In vivo, the combination of Shh and Wnt signalling determines the differentiation of dorsoventral neuronal types in the developing midbrain (Li et al., 2008). The in vitro application of Wnt/β-catenin ligand, Wnt-7a and Shh to NS/PCs from the PVZ led to an increase in neural phenotype subpopulations. Wnt-7a switches the differentiation process from gliogenesis to the neural lineage and enhances the outgrowth of developing processes in the early stages of the differentiation process. On the other hand, Shh promotes the

proliferation of neonatal NS/PCs during the entire process of differentiation, but Shh has no influence on the outgrowth of developing neurites (Prajerova et al., 2010). The group of morphogens together with trophic factors not only play important roles in the early development of the neuronal ectoderm, but they are also critical for adult neurogenesis and gliogenesis. For example, the combination of FGF8 and Shh is required for ESC differentiation toward a dopaminergic phenotype (Yan et al., 2005)

2.4.3. Mesenchymal stromal cells

Among the very wide variety of stem cell types available for therapy, mesenchymal stromal cells (MSCs) are particularly attractive because, compared to other stem cells, they are easier to isolate and expand from patients without serious ethical or technical problems. MSCs were first described as bone marrow stromal cells responsible for stromal reconstruction during the growth process (Hwang et al., 2008). However, later it was shown that MSCs have a variety of functions, including the release of soluble factors supporting hematopoiesis and vascularisation; they can also differentiate into bone, cartilage and fat tissue cells *in vitro* (Cho et al., 2009).

Initially, it was expected that MSCs will be able to replace missing neural cells after transplantation into the injured spinal cord (Parekkadan and Milwid, 2010). However, according to electron microscopy, fewer than 3% of this type of stem cell modifies their cytoskeleton so as to resemble neurons morphologically, but they do not undergo "true" transdiffrentiation – a process by which cells of one organ lineage generate cell types from different organs (Jendelova et al., 2004). Paracrine and trophic effects are more evident after the transplantation of MSCs into injured nervous tissue (Parekkadan and Milwid, 2010). After being transplanted into animal models of SCI, MSCs can migrate into the surrounding tissue, enhance lesion repair, stimulate axonal regeneration across the lesion site, and improve functional recovery (Lee et al., 2007 B). Despite their low proliferative and differentiation potential, MSCs are frequently used in experimental studies and clinical trials. The results of several such studies have already been published (Sykova et al,. 2006; Lee et al., 2011), and many other studies are ongoing worldwide.

2.4.4. Neural stem cells

Neural stem cells (NSCs) are the largest group of stem cells suitable for transplantation in SCI. NSCs were initially derived from the fetal or adult CNS, which determines their features, proliferative capacity and ability to differentiate. In vitro, NSCs are capable of differentiating into any type of functionally active neural cell: neurons, astrocytes or oligodendrocytes (Cai and Grabel, 2007; Cao et al., 2002), except for restricted progenitor cell lines, which are committed to differentiation into a specific cell phenotype (Ronaghi et al., 2009). An undesirable effect of some NSC lines in contrast to that of ESCs is that transplanted in vivo, NSCs give rise almost exclusively to astrocytes and to only a relatively few oligodendrocytes and occasional neurons, a situation thought to be responsible for neuropathic pain such as allodynia. Nevertheless, several studies have demonstrated functional improvement and even motor recovery after the transplantation of different types of NSCs into animal models of SCI (Kim et al., 2009; Lee et al., 2009; Lu et al., 2003; Pan et al., 2008;). In the last several years, an important new approach of combinating NSCs with biocompatible hydrogels has become more popular and is yielding very promising results (Brannvall et al., 2007; Teixeira et al., 2007; Thonhoff et al., 2008).

2.4.5. Induced pluripotent stem cells

Induced pluripotent stem (iPS) cells – pluripotent stem cells dedifferentiated from adult somatic cells – have appeared as an alternative to ESCs as a source of pluripotent stem cells. Initially, iPS cells were derived from mouse fibroblasts by introducing four genes – *Pou5f1* (also known as *Oct3/4*), *Sox2*, *Klf4* and *c-Myc* – into the cells via retrovirus-mediated gene transfer (Takahashi and Yamanaka, 2006). Subsequently, iPS cells were derived not only without using *c-Myc*, which is known to be an oncogene, but even without using genetic alteration, thereby allaying concerns regarding oncogene reactivation and tumor formation. Protocols similar to those for ESC differentiation can be used for deriving the required neural cell phenotype from iPS cells. A major advantage of iPS cell-derived precursors compared to ESCs is that the use of these cells for transplantation avoids both graft rejection and also ethical problems. However, further examination of iPS cells is needed to determine their safety and efficacy for subsequent transplantation (Okano H, 2010; Hu et al., 2010).

2.4.6. Genetic modifications of stem cells and stem cell factor production

Controlled genetic manipulation with stem cells brings new opportunities not only for SCI treatment, but also for cell therapy in general (Kuegler et al., 2010). For example, the immortalization of neural fetal neural precursors with c-mycER^{tam} keeps the cell line clonal and rapidly proliferating in the presence of the synthetic drug 4-hydroxy-tamoxifen (4-OHT). However, in the absence of 4-OHT, cell growth is arrested and the cells differentiate into neuronal and astrocytic phenotypes (Pollock et al., 2006). The same approach was successfully used for immortalizing several stem cell lines (Lee et al., 2007 A).

Besides their proliferative capacity, which is very impotent for successful and reproducible treatment in cell replacement therapy, stem cells serve as a source of a number of trophic factors capable not only of assisting the reconstruction of damaged tissue, but even of stimulating vascularisation processes (Rossi and Keirstead, 2009) and modulating the immune response by suppressing inflammatory mechanisms (Lee et al., 2008). Each type of stem cell produces a different set of trophic factors. Not only MSCs can serve as a source of paracrine stimulation. NSCs grafted into the lesioned nervous tissue can produce factors with neuroprotective effect such as NGF, BDNF, GDNF, NT3 (Lu et al., 2003) or VEGF (Kim et al., 2009). Unfortunately, naive MSCs and NSCs produce such trophic factors in low and thus ineffective concentrations. Modern approaches of molecular biology allow the genetic modification of stem cells to enhance specific molecule expression and support the regeneration and differentiation of endogenous tissue.

2.4.7. Glial cell progenitor therapy

A number of studies have suggested that the failure of the CNS to regenerate is due to the hostile environment of the injured spinal cord rather than to a limitation in the growth of axons. In order to achieve complete system recovery, the remyelination of spared axons and the myelination of newly formed axons through treatment combining NSCs and adult glial cells are necessary, not just the pharmacological stimulation of reactive astrocytes and the blockade of oligodendrocytes (Kumagai et al., 2009).For example, the application of glial-restricted progenitors (GRPs) or oligodendrocyte precursor cells (OPC) together with Schwann cells and olfactory ensheathing cells promotes white matter regeneration in animal models of genetic or chemical demyelination (Reier P.J., 2004). Human GRPs migrate into the lesion and adopt a mature glial phenotype. Animals treated with hGRPs together with

Schwann cells showed significantly better electrophysiological conductivity compared to control animals (Walczak et al., 2011). OPCs derived from hESCs display typical OPC marker expression: Olig1 (90%), NG2 (80%) and PDGFRα (70%). After the application of OPC and Schwann cells in a rat model of contusion cervical injury, OPC migrated into the lesion, terminally differentiated into oligodendrocytes and were associated with a positive physiological outcome in forelimb function recovery (Sharp et al., 2010). Schwann cells have been shown to be helpful as bridging and supporting cells, and together with neural stem cells are bringing promising results (Carroll et al., 1997; Jones et al., 2001; Oudega et al, 2005)

2.5. Hydrogel material

Hydrogels are hydrophilic polymers defined by their ability to reach very high water content. Hydrogels have high swelling ability, up to 98% of their volume, and have a porous structure with a pore size generally ranging from 10um to100 um in diameter (Lesny et al., 2002, 2006; Pradny et al., 2005, 2006). The basis for such an ability of hydrogel materials consists in distinctions between different types of water. The incorporated water is divided into the primary bound water around hydrophilic and charged structures, the secondary bound water surrounding hydrophobic structures and the free (bulk) water filling the rest of the hydrogel (Hoffman et al., 2002).

2.5.1. Hydrogel materials categories

As written above, biocompatible materials, including hydrogels, are divided into several groups according to their origin, composition, degradability and the possible direction of cell growth (tab.4.). Starting with their composition and origin, they are divided into natural implants [polysaccharides and their modifications (chitosan), fibronectin, hyaluronic acid or collagen type I] and synthetic biodegradable implants [including PEG, Matrigel, fibrin glue or p(α-hydroxy acids such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA)] on one side, and synthetic non-degradable implants [including HEMA and HPMA (neurogel)] and composite hydrogel materials on the other side (Nisbet et al., 2008; Novikova et al., 2003).

In addition to classification based on the origin of the material, its degradability and axonal guidance, another classification into physical and chemical hydrogels is used. Physical hydrogels are held together by molecular entanglement and ionic strength, or weak force interactions such as H bonding or hydrophobic forces. Networks of polymeric molecules are irregular, with long chains of different lengths. Physical hydrogels are also called reversible or ionotrophic. The physical hydrogel group includes materials such as calcium alginate, polysaccharides, biotin etc... Changes in pH or ionic strength dissolve these materials (Guan et al., 2008). Chemical hydrogels such as HEMA, HPMA, PAN or PEG based on covalently-crosslinked networks are, on the other hand, more stable. MA-materials serve as a crosslinker, for example. Chemical hydrogels are also called permanent hydrogels. Both types of materials, physical and chemical hydrogels, create irregular clusters and contain intrinsic pores of different sizes (Hoffman et al., 2002).

2.5.2. Hydrogel features for mimicking endogenous tissue

Thanks to chemical modifications, changes in water content and pore modulation, hydrogels can imitate almost any tissue of the animal or human body. Differences in water content and polymerisation subunits allow hydrogel materials to imitate almost any tissue ECM in terms of their surface wrinkles, degree of stiffness and structure. Hydrogels are mostly used to imitate soft tissue. For example, the degree of stiffness of HEMA or HPMA is similar to that of the postnatal environment in the CNS (Nisbet et al., 2008; Lesny et al., 2002), an observation that led to the idea of bridging CNS injuries. Contact lenses have been known for a long time, but the supplementation of the endothelial cell environment stimulates revascularisation (Moon et al., 2010) while modifying the subunits with SiO_2 molecules for hard tissue implants in bone tissue engineering (Costantini et al., 2008) leads to the idea of whole organ and tissue reconstruction.

2.5.3. Chemical modifications

Hydrogel materials and other biocompatible polymers have been tested for more than ten years as either simple or composite materials, with the aim of encapsulating growth factors and/ or cells. One of the modern composite material strategies consists of different shell-core domains opening their internal capsule only at a specific temperature or pH. This strategy serves to prolong trophic factor activity (Brandl et al., 2009; Hyatt et al., 2010; Ranjha et al., 2010; Yu et al., 2010). Another method for trophic factor release in a gradient concentration has been designed by Y. B. Lee and coworkers. The collagen and fibrin parts of the hydrogel were separated in their material, and the collagen part hosted murrine neural precursor cells. The fibrin part of the gel either did or did not contain VEGF. The control group had both the cells and the growth factor in the collagen part. Only the group of animals receiving gels with separated trophic factor and cells showed cell guidance, even though the factor was present in two of the three experimental groups. Without separation, the factor was active but did not serve as guidance molecule (Lee et al., 2010 B).

These applications are opening new fields for hydrogel use. Hydrogels are not only passive materials, simply bridging the lesion gap, but they also can create an active environment and thus stimulate donor and endogenous cell repair mechanisms to bypass the lesion (Sung et al., 2008; Wei et al., 2010; Woerly et al., 2004).

2.5.4. Stem cell culturing

The idea of culturing stem cells on 3D biomimetic artificial materials came in successive steps. With the aim of creating a suitable environment for stem cell proliferation and spreading, several methods to increase the attractiveness of the cell substrate have been developed. Coating the substrate with supportive cells, creating a surface similar to the ECM in terms of charge structure (Pradny et al., 2005), hydrophilic surfaces (Nisbet et al., 2006) and the activation of membrane receptor cascades (Michalek et al., 2005; Shu et al., 2003; Zhu et al., 2009) have all shown a positive effect on cell fate. From there, a further step leads to the idea of a 3D material more similar to the natural environment and using internal channels to guide axons through the hydrogel material. The 3D material supports differentiation into an adult cell phenotype (Brannvall et al., 2007; Jongpaiboonkit et al., 2009; Hejcl et al., 2009). A series of *in vitro* and *in vivo* tests were started in order to search for a suitable combination of hydrogel material and cells. In order to find the right matrix, whole ranges of materials have been tested (Bergethon et al., 1989; Khetan et al., 2009; Teixeira et al., 2007; Thonhoff et al., 2008). In the case of brain and spinal cord injury, the application of hydrogel materials shows the ability to increase the smoothness of the spinal cord / hydrogel border and also to reduce glial scar and cavity formation. The infiltration of neurons and glial cells has been observed (Duconseille et al., 1998; Hejcl et al., 2008 A; Hejcl et al., 2009).

2.5.5. Natural materials

Natural materials, together with soluble factors and supportive cells, create an interesting system able to increase regeneration after CNS injury (Novikova et al., 2006; Novikov et al., 2002; Willerth et al., 2007). Their implantation can be simpler, for example, when using injectable self-polymerising scaffolds (Guan et al., 2008; Zhou et al., 2009). Natural material, or materials synthesised from natural compounds including collagen type I, elastin, alginate, or fibrin, are often a component of the endogenous ECM, and thus modifications by ECM primary sequences are not necessary (Fig.4). For their modification, composites or copolymers are used (Novikova et al., 2003; Shu et al., 2003). Most biocompatible materials, although their supporting abilities have been known for years, are still the subject of intense interest thanks to new technologies that utilize them in novel ways. The grafted cells show diverse reactions depending on the type of material. In the work of Lyudmila Novikova, alginate hydrogel (with/without fibronectin) and matrigel were used together with OECs, SCs or BMSCs to form a supportive environment for mouse dorsal root

ganglion (DRG) neurons. The results showed that alginate hydrogel supports cell survival, but in an abnormal spherical shape, and halts cell proliferation. Following the incorporation of a fibronectin modification, only OECs were proliferative, but DRG neurons were in both cases unsupported. In contrast, in the matrigel environment the supporting cells proliferated and the axons of DRG neurons were prolongated (Novikova et al., 2006). It has been shown that the application of artificial polymeric scaffolds and their combination with NSCs can have a positive effect on decreasing the glial scar and reducing the tissue loss caused by secondary injury processes as well as helping sensory-motor recovery after SCI (Teng et al., 2002).

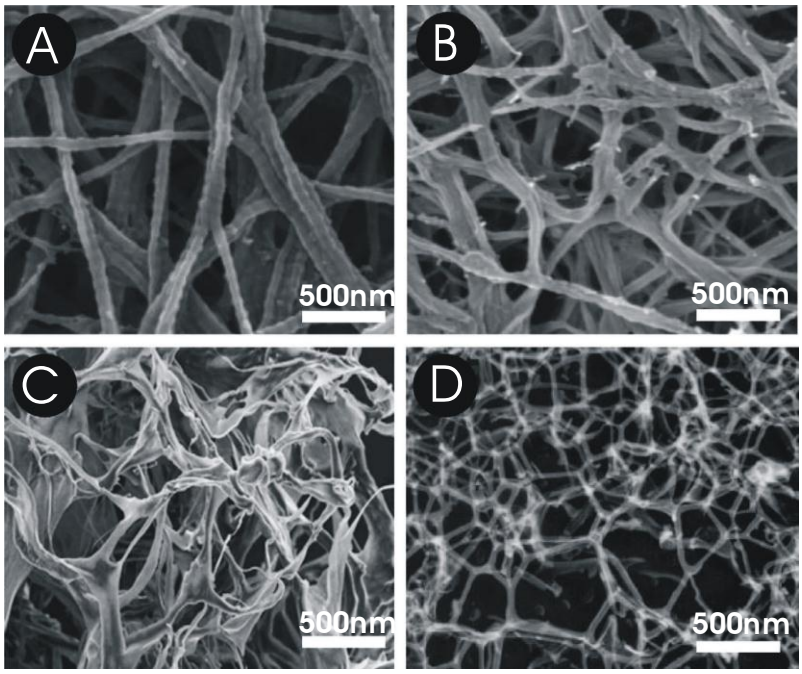


Fig.4. Natural hydrogel materials. Scanning electron microscope images of biologically derived and biologically produced hydrogels: Fibril structure of collagen type I (A), fibrin network (B), cross-linked adipic acid modified hyaluronic acid hydrogel(C) and self-assembling peptide RADA16-II (D) hydrogel (Patterson et al., 2010).

2.5.6. Synthetic materials

The application of synthetic materials mimicking the endogenous tissue has a long history. Contrary to natural or natural-based materials, most of the synthetic polymers alone are less suitable in terms of cell proliferation or survival. However, their advantage is their easy modification with a surface charge or adhesion molecules. Thanks to modifications based on the type of subunits, pore structure and surface features, synthetic hydrogels offer a variety of stable structures with different characteristics. In tissue engineering for SCI treatment, HEMA, HPMA and PEG hydrogels are used the most (Fig.5). PEG hydrogels less supportive of cell growth than HEMA and HPMA hydrogels, but often serve as capsules for growth factors or their combination with cells (Brandl et al., 2010; Lieb et al., 2005; Lin et al., 2009; Zhu et al., 2009). Between HPMA and HEMA hydrogels, there are also differences. Unmodified HPMA hydrogel is more biocompatible than HEMA material. HPMA hydrogels have been shown to reduce cavity formation, promote axonal growth through the hydrogel and improve functional regeneration (Woerly et al., 2001; Woerly et al., 2004). However, both the biocompatibility and attractivity of HEMA can be markedly increased through modifications (Bergethon et al., 1989; Hejcl et al., 2008 A; Kubinova et al, 2010; Kubinova et al., 2009; Michalek et al, 2005).

2.5.7. Degradable materials

Degradable materials, both natural (Novikov et al., 2002) and synthetic, (Pradny et al., 2006) are used as a consequence of the idea that partially stable bridging materials accelerate the onset of regeneration and that such materials are able to degrade in the injured area into nontoxic metabolites. It has been shown that a long term effect of the bridging material is necessary to enhance regeneration, but more and more materials with longer-lasting stable structures are being developed. Numerous studies have shown the positive effect of biodegradable implants (Ashton et al., 2007; Sahoo et al., 2008).

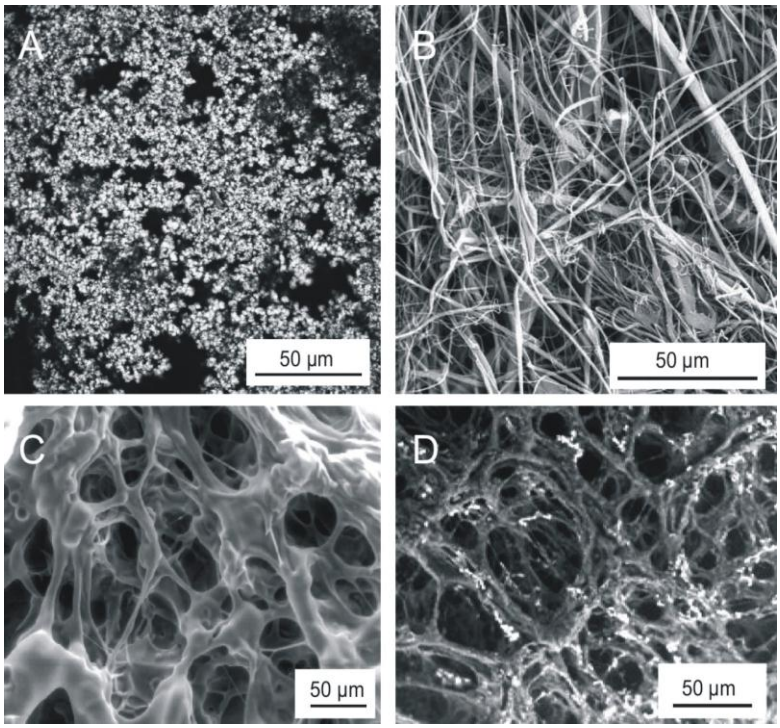


Fig.5. Synthetic hydrogel materials. Four types of hydrogels implanted in experimental SCI in our Centre. A. HPMA-RGD hydrogel. B. Nanofibre scaffold. C. HEMA hydrogel. D. Biodegradable HEMA-based hydrogel partially degraded after 2 days (Hejcl et al., 2008 B).

2.5.8. Pore structure

Channel structures and their modification for the functional connection of the spinal cord stumps and axonal, glial and blood vessel penetration through the whole hydrogel volume are necessary. Biomimetic materials with varied channel structures could have selectivity for different types of cells. For example, with a suitable combination of material and pore structure, a stabilized vascular network can be created directly by mimicking the endogenous environment (Moon et al., 2010). The combination of human microvasculature endothelial cells with an HEMA hydrogel containing PEG led to the creation of an effective vascular system due to the resulting sponge lattice-type structure with greater pore interconnection (Dziubla and Lowman, 2004). Pore linearity and suitable trophic factor release from the implanted material have been shown to be important for axonal growth, bridging the lesion (Stokols and Tuszynski, 2004). The structure of the pores is influenced by the type of polymerisation and subunits. Pores can be simply created by the intersubunit space (as in the case of HPMA hydrogels), or by using crystallization methods (HEMA, hyaluronic acid hydrogel). One example of the possible design strategies is the combination of HPMA subunits and the crystallization method of pore structuring in macroporous HPMA gels. Pores creating a network for dendritic growth are one of the methods using the crystal templating technique to promote oriented cell growth and cell anchorage (Zawko et al., 2010).

2.5.9. Surface modifications to increase hydrogel biocompatibility

Surface qualities such as shape (Guvendiren et al., 2010), charge (Pradny et al., 2005), or primary sequences of growth factor, guidance or ECM molecules (Aizawa et al., 2008; Kubinova et al., 2010; Lieb et al., 2005) have led scientists to investigate which material can best serve for promoting tissue regeneration and axonal sprouting in spinal cord regeneration .

In vitro tests of different scaffold materials have shown that besides material subunits (collagen, elastin, HPMA, HEMA, PEG…), the swelling ratio and surface morphology (Guvendiren et al., 2010), resulting in cell attachment, another surface modification is necessary to enhance cell proliferation. The necessity of modification stems from the idea of using hydrogel materials to mimick features of ECM molecules, which intermediate one of the signals necessary for cell spreading (Rao and Winter, 2009).

The type of chemical bond used to incorporate molecules on a material's surface is very important. The most frequently used approach to modification is the covalent binding of factors, which enables the long term coupling of the factors. This type of bond is suitable for ECM molecules or other insoluble factors. Smelting procedures are intermediate in terms of bond strength between a strong covalent bound and an ionic or H bond. Smelting allows for the gradual release of the bound molecules and is thus convenient for the long term release of soluble factors.

2.5.9.1. Surface charge

One of the first tested surface characteristics was the influence of charge. On HEMA hydrogels, MSCs were cultivated *in vitro* and *in vivo* under different surface charge conditions. The following four groups of materials were tested: hydroxy-ethyl-methacrylate-[2-(methacryloyloxy)ethyl] trimethylammonium chloride (HEMA- MOETA-$Cl^{+)}$, hydroxy-ethyl-methacrylate –sodium methacrylate (HEMA-$MA^{-)}$, HEMA-$MOETACl^{+}$-MA^{-} terpolymer and polyelectrolyte complex of HEMA-MA^{-} with $MOETACl^{+}$ copolymer (Pradny et al, 2005). The highest cell density was observed in the positive charged HEMA hydrogels. However, the distribution of cell density was unequal, and a higher density was found at the hydrogel border. A lower cell density, but equally dispersed on the hydrogel volume was obtained in the negative and tetrapolymeric HEMA complexes. The lowest cell density was observed in the polyelectrolyte complex, probably due to hidden surface charges (Lesny et al., 2006). In an *in vivo* model of spinal cord injury, positive charged functional groups promoted

connective tissue infiltration and extended axonal regeneration inside the hydrogel bridge (Hejcl et al., 2009).

2.5.9.2. ECM primary AA sequence modifications

The translocation of information via integrins, the p75 receptor and other receptor molecules into the internal cellular signal cascade is necessary to increase cell survival, growth, proliferation, and differentiation. Cell-cell and/ or ECM contact stimulates features of the cell environment to signal for cell survival, proliferation, specific gene transcription including axonal pruning and/or cell death (Vanderhaeghen et al, 2010; Yu, 2005; Woerly, 2004). Because of the difficulty of modifying materials with complete protein structures, the anchoring of important primary sequences of AA or non-protein molecules is often used (Gonen-Wadmany et al., 2007; Jia et al., 2009). The combination of AA primary sequences and non-protein structures such as cholesterol molecules applied with laminin on superporous HEMA hydrogels increases support for stem cell growth and proliferation compared to the use of only one of the factors (Kubinova et al., 2009). A few primary AA sequences for hydrogel surface modification are used the most; among them the RGD tripeptid sequence is the most popular. It mimics the integrin substrate fibronectin binding site. PEG, Poly (ethylene glycol) diacrylate (PEGDA), and HPMA modified with the RGD or cRGD sequence showed promising results in acute and chronic spinal cord injury treatment (Hejcl et al., 2010; Zhu et al., 2009). Laminin-derived IKVAV and YIGSR peptides are the next most used primary sequences. As a result of the IKVAV AA primary sequence, covalently modified hyaluoran or macroporous HEMA hydrogels promote the migration, adhesion, differentiation and axonal pruning of implanted human fetal neural precursors (SPC-01) and endogenous precursors *in vitro* and *in vivo* (Kubinova et al., 2010; Wei et al., 2007).

2.5.9.3. Trophic factors and other molecules for surface modification

In addition to the primary AA sequences of ECM molecules, which bind integrin receptors, (fibronectin, laminin, collagen, etc.), other molecules including growth factors, neurotransmitters and neuromodulators are often used to modify the hydrogel surface (Tessmar et al., 2007). These approaches to surface modification provide diversity in a number of applications. Hydrogel materials can be modified to modulate surrounding cells and spinal tissue. Modulation by combining a natural ECM-imitating structure together with poly L-lysine and a Nogo 66-receptor antibody, not only influences the cells spreading into the material, but also produces environmental changes in astrogliosis (Wei et al., 2010).

Moreover, the application of PEG hydrogel containing anti-TNF-α has shown an anti-inflammatory effect, increasing the probability of implanted cells surviving in SCI treatment (Lin et al., 2009). On the other hand, hydrogel materials can influence their own internal characteristics to promote ingrowth. However, not every combination of trophic factor and type of bond is guaranteed to improve the hydrogel environment. In Aizawa's experiments, in which he and his coworkers utilized platelet derived growth factor (PDGF) as a covalent surface modification of hydrogel, they showed that the type of binding of the molecules plays an important role in the promotion of proliferation. The use of soluble factors covalently bound to the hydrogels required concentrations of the molecules three times higher than those required when using molecules that are naturally covalently bound (such as ECM protein primary AA sequences or receptors) (Aizawa et al., 2008). Through a combination of specific surface modifications and material subunits, the resulting material can be selective for supporting specific stem cells (Jung et al., 2009).

2.5.10. Hydrogel conclusions

These examples show that hydrogel materials can be modified to modulate the intrinsic environment for cell growth or to decrease the inhibitory influence of the extrinsic environment surrounding the lesion. Hydrogels fulfil the requirements of a convenient graft material with an easily modifiable structure. With proper adjustment of their physical and chemical properties, they can mimic the CNS environment (Hejcl et al., 2008 A; Nisbet et al., 2008; Lesny et al., 2002). Along with evolving cell therapy (Rauch et al., 2009; Sykova, 2006 B), trophic and protective factor application (Lee et al., 2010 B; Wei et al., 2010) and signal sequence anchoring (Wei et al., 2007), hydrogels can serve for preclinical studies and hopefully for the treatment of patients with spinal cord injuries.

3. Aims of the work

The aim of the work was to evaluate the effect of treating a spinal cord hemisection, as an experimental model of SCI, by a combination of human fetal neural precursors (SPC-01 cell line) and P2544-1 hydrogels.

1) To compare the *in vitro* and *in vivo* growth, proliferation and differentiation of SPC-01 fetal neural precursors on P2544-1 hydrogels.

2) To analyze functional improvement after the transplantation of a P2544-1 hydrogel seeded with SPC-01 cells.

3) To evaluate the influence of fetal neural precursors and the P2544-1 hydrogel on tissue atrophy, astrogliosis, revascularisation, and axonal sprouting in the damaged spinal tissue.

4. Material and methods

4.1. Animals

Two-month-old male Wistar rats (n= 31) (Velaz, Prague, Czech Republic) with body weight between 300–330 g were used. The animals were separated into two groups: the control group (n= 21), which underwent spinal cord hemisection surgery with no additional treatment except antibiotics (Gentamicin), immunosuppression (Cyclosporine A) and corticosteroids (MPSS), and the treatment group, which underwent spinal cord hemisection followed by the implantation of a P2544-1 hydrogel seeded with SPC-01-derived cells and the same pharmacological substances as used in the control group (Gentamicin, Cyclosporine-A, MPSS). This study was performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Central Commission for Animal Protection of the Academy of Sciences of the Czech Republic in Prague

4.2. P2544-1 hydrogels

Serotonin-containing (serotonin hydrogen oxalate) hydroxy ethyl methacrylate hydrogels (P2544-1) were prepared in the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic. The optimal concentration of the serotonin-containing molecules anchored on the HEMA hydrogels was determined in a preliminary study (data not yet published). Before application, the hydrogels were stored in phosphate buffered saline (PBS pH 7,4) at +5 °C.

4.3. SPC-01 culture

The SPC-01 line was derived from human somatic stem cells from 8-week-old fetal spinal cord tissue and modified with the conditionally immortalizing gene c-mycER^{TAM}. The product of the c-mycER^{TAM} gene in the presence of 4-hydroxy-tamoxifen stimulates cell proliferation. In the absence of 4-hydroxy-tamoxifen, cell growth arrests, and the cells differentiate into neurons and astrocytes (Pollock et al., 2006). The SPC-01 line was cultured in DMEM/F12 supplemented with 20% human albumin, 50mg/ml apotransferine, 8,1mg/ml putrescune DiHCL, 10 mg/ml human recombinant insulin, 20 μg/ml progesterone, 200 mM L-glutamine, 20 μg/ml sodium selenit, 10 μg/ml hrFGF, 10 μg/ml hrEGF,

penicilline/streptomycine at 50U/ml (GIBCO) and 1 mM 4-hydroxy-tamoxifen as an anti-apoptotic factor. SPC-01 cells were seeded on HEMA hydrogels. The gels with the cells were cultured for 28 days. The medium was changed every two days, and every seven days several samples of the gels with cells were chosen for immunohistochemical analysis. After 28 days the gel implants were used for implantation. In some experiments SPC-01_GFP3 cells (transfected with a GFP construct) were used.

4.4. Grafting in an acute SCI hemisection model

Hemisection and hydrogel implantation were performed under total anaesthesia. For anaesthesia, an intraperitoneal (i.p.) injection of 10 mg/ml pentobarbital was administered at a dose of 6 ml/kg body weight. Antibiotics (gentamicin, 0,05ml intramuscular) were administered before surgery. To protect against Salivation, 0,2ml of atropine was injected subcutaneously (s.c.). The fur in the thoracic spine area was removed and local anaesthesia was applied (mesocain 0,3ml s.c. and i.m), because of the limited analgesic effect of pentobarbital. The animal was fixed by its hindlimbs to the surgical table during the operation.

The surgery was carried out using an operating microscope (Zeiss) at 15-25xs under aseptic conditions. The hemisection was performed at the level of Th8-9. A 2-3cm long incision was made in the midline, and then the fat tissue was removed. After separating the paraxial muscles, the processi spinosi Th8-9 were removed. Then a 2 mm length of dura mater was separated from the midline to the right edge of the spine, followed by hemisection. During the hemisection procedure, a cavity 2x2x2 mm in size was created. In order to maintain homeostasis, it was necessary to keep the cavity clean and to prevent hematoma creation, the resorption of which can later lead to pseudocyst formation. The bridging material was implanted into the prepared cavity. The hydrogel was adjusted to fit the shape and size of the cavity so as not to traumatize the tissue or create pseudocyst cavities in the space between the tissue and the hydrogel.

4.5. Postoperative care

The animals were injected with ampicilin i.m. (50mg/kg, Ampicilinum natricum, Biotika) twice a day for the first few days after surgery. During the experiments, cyclosporin A (10mg/kg, Sandimmun, Novartis) and MPSS (1,7mg/kg, solu-medrol, Pfizer) were injected once a day; after one month, the frequency decreased to once every two days. The animals

were housed in pairs in internally ventilated cages (IVC, Tecniplast) during the experiment to prevent infections.

4.6. Behavioural testing methods

4.6.1. BBB test

The BBB test is the most commonly used locomotor test worldwide. The scoring scale ranges from 0 (no observed hindlimb movement) to 21 (completely normal movement-coordinated gait, parallel paw position, tail consistently up). The scale is divided into smaller parts; the first part, from 0 to 7, focuses on movement of the hindlimbs, from no observed movement (0) to the rapid movement of all three joints (7). The second part, from 8 to 13, ranges from weight unsupported plantar steps through weight supported dorsal / plantar steps and the coordination of the forelimbs and hindlimbs. The third and final part from 14 to 21 requires more detailed observation, concentrating on the plantar position during movement, the use of the tail and trunk stability (each score is described in detail in the accompanying table). Each hindlimb joint is watched and the individual movements analyzed; Pretraining of the animals is not necessary. One problem associated with BBB testing is the subjective input of the testers. In order for the test to be more objective, two independent "blind" testers do the scoring. The BBB scale is more detailed in the higher scores than in the initial lower scores. This problem can be solved by sub-scoring, which means that the final score is determined from the details of the individual visible movements and adjusted up or down accordingly (Basso et al., 1996).

4.6.2. Plantar test

The plantar test belongs to the group of hot plate tests. It is standardized and used for measuring changes in thermal sensitivity after SCI. Three rats are placed into separated plastic cages with a thermoplastic floor, and each animal is tested five times for each hindlimb. Under the Plexiglas floor there is a movable infrared generator, with the cut-off time usually set to 35s and the intensity set between 50 and 60 units, corresponding to the midpoint of the emission range. These settings prevent any injury to the tested paw. The measured variable is the time elapsed between the start of infrared heat emission and the animal's reaction to the thermal stimulus. In order to assure that the time interval between two consecutive tests remains the same, the animals are tested in the sequence (1, 2, 3, 1, 2, 3...). It is crucial to always test the same part of the paw due to the different concentrations of thermal receptors at different locations in the paw.

Tab.2. Basso, Beatie and Bresnahan locomotor score table

BBB value	
0	No observable hindlimb movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e. when stationary) or occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps; no FL–HL Coordination
11	Frequent to consistent weight-supported plantar steps and no FL–HL coordination
12	Frequent to consistent weight-supported plantar steps and occasional FL–HL coordination
13	Frequent to consistent weight-supported plantar steps and frequent FL–HL coordination
14	Consistent weight-supported plantar steps; consistent FL–HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL–HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL–HL coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
17	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift-off
18	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
19	Consistent plantar stepping and consistent FL–HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact and lift-off, and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift-off, and trunk instability; tail consistently up
21	Consistent plantar stepping and consistent gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

Legend: BBB locomotor scores, which evaluate each hindlimb movement and describe functional improvement after SCI (Basso et al., 1996).

4.7. Histological processing and immunohistochemical staining

Rats were sacrificed three months after surgery. The anesthetised animals were perfused with 4% paraformaldehyde in 0,1MP PBS (pH 7,4). After perfusion, a 5 cm piece of the spinal cord containing the centre of lesion was removed from the body, and the spinal cord, along with the bone, were left in 4% paraformaldehyde in 0.1 M phosphate buffer overnight. The fixed spines were immersed in PBS with 30% sucrose. Frozen spinal sections (40um) were cut through the area of interest. To identify SPC-01 cells in the spinal cord, antibodies directed against βIII-tubulin (S- Sigma T-8660), nestin (Chemicon MAB5320) and glial fibrillary acidic protein (GFAP- Cy3 Sigma C-9205) were used. To identify the influence

of the implanted cells and the hydrogel on the surrounding lesioned tissue, hematoxylin-eosin staining and antibodies directed against GFAP (GFAP- Cy3 Sigma C-9205), neurofilaments 160 (Sigma N5264), and the endothelial cell marker RECA1 (abcam ab9774) were used. To visualize primary antibody reactivity, appropriate secondary antibodies were used: goat anti-mouse IgG conjugated with Alexa-Fluor 488 or 594 and goat anti-rabbit IgG conjugated with Alexa-Fluor 594 (molecular probes, Invitrogen, A11029, A11032, and A11012). The samples were washed three times with PBS and mounted with vectashield (Vector H-1000) on a glass slide.

4.8. *In vitro* immuhistochemical staining

Cells seeded on hydrogel pieces were washed in PBS (pH 7,2) and fixed with 4% paraformaldehyde in PBS for 30 minutes. Prior to immunostaining, the fixed cells were twice washed in PBS. Permeabilisation and blocking were carried out in a blocking buffer consisting of 0,1% Triton (Chemicon 2170-S), 5% goat serum, and 1mg/ml bovine serum albumin in Tris buffer for 20 minutes. To identify SPC-01 cells and differentiated cell markers, antibodies directed against neuronal-cell adhesive molecule (N-CAM Chemicon AB5032), neurofilaments 70 (Chemicon MAB1615), nestin (Chemicon MAB5320), synaptophysin (Chemicon MAB5258) and βIII-tubulin (S- SigmaT-8660) were used. To visualize primary antibody reactivity, appropriate secondary antibodies were used: goat anti-mouse IgG conjugated with Alexa-Fluor 488 or 594 (molecular probes, Invitrogen A11029, A11032). The samples were washed three times with PBS and mounted with vectashield (Vector H-1000) on a glass slide.

4.9. Fluorescence and confocal microscopy

The samples were examined using a spectral confocal microscope (LEICA TCS SP) equipped with an Ar/HeNe laser or a ZEISS AXIO Observer D1 microscope (Carl Zeiss, Germany). For confocal microscopy, 405nm (DAPI), 560nm (gamAF594), and 488nm (Ar, gamAF488) lasers were used to visualize the neural markers expressed by differentiated SPC-01 cells *in vitro* and *in vivo*. The Observer D1 microscope was used to visualize the quantifiable effects of the cell and hydrogel implant on the surrounding damaged tissue.

4.10. Marker analysis

For evaluating spinal tissue atrophy, the surface areas of the tissue on hematoxylin-eosin slices were averaged using image J software; the analysis was performed at 25X magnification. The area occupied by the hydrogel in the treated group was not counted towards the width of the spinal tissue in the centre of the lesion.

For evaluating the degree of astrogliosis in the tissue surrounding the hydrogel, an analysis based on the size of the area displaying high fluorescence intensity for GFAP positivity was made at 200X magnification. Five specific locations surrounding the main lesion cavity were measured to determine the extent of GFAP positivity. The measurements were carried out on five slices from the spine of each treated and control animal that survived to the end of the experiment.

For evaluating the degree of axonal degeneration and the extent of axonal sprouting into the hydrogel, an analysis based on the size of the area displaying high fluorescence intensity for NF160 positivity was made at 400X magnification. Five specific locations were measured to determine the extent of NF160 positivity. Four measurements were made on the side of the hemisection and a fifth one on the side opposite to the hemisection in the centre of the lesion. The measurements were made on five slices from the spine of each treated and control animal that survived to the end of the experiment.

For evaluating the extent of revascularisation in the hydrogel, an analysis based on the size of the area of high fluorescence intensity for RECA positivity was made at 400X magnification. Five specific locations surrounding the main lesion cavity and filled with hydrogel material were measured to determine the extent of RECA positivity. Five slices from the spine of each treated and control group animal that survived to the end of the experiment were analyzed. All of the fluorescent analyses were carried out using Axiovision 4.8 software (Zeiss).

4.11. Statistical analysis

For evaluating the behavioural data, each week's data from control and treated animals were tested for variability using the F-test. For evaluating significant differences between the groups of animals in each testing procedure, a t-test (two sides, two choices, for the same variability data) was used, while in the case of nonparametric data, the t-test for unequal variability was employed. To better visualize trends in the data over time, graphs were constructed, while linear regression was applied to the plantar test results for both hindlimbs. The standard error of the mean was calculated in order to better assess data

variability. For immunohistochemical analysis, the F-test was used to determine variability within the groups. For evaluating the statistically significant differences between the treated and control groups, a t-test (two sides, two choices) was used.

5. Results

5.1. SPC-01 proliferation and differentiation

5.1.1. SPC-01 culture

We performed an immunohistochemical analysis to evaluate the compatibility of SPC-01 cells and P2544-1 hydrogel *in vitro*. After 28 days of culturing, the population of GFP-human fetal NSCs (SPC-01) on the P2544-1 hydrogel was nearly confluent. Despite the presence of 4-OHT factor, human fetal NSCs differentiated into a neural phenotype. Cells were positive for several markers of neural differentiation, including neurofilaments 70 (Fig.6 B, C), neuronal-cell adhesive molecule (Fig.6 A), nestin (Fig.6 D), synaptophysin (Fig.6 E) and βIII-tubulin (Fig.6 F). These results show the high biocompatibility between the SPC-01 cells and P2544-1 hydrogel *in vitro*. Serotonin-containing molecules serve as a good attractant and can partially affect neuronal differentiation.

5.1.2. SPC-01 proliferation and differentiation in vivo

To determine SPC-01_GFP3 cell destiny and to study the compatibility of SPC-01 cells and P2544-1 hydrogel *in vivo*, an immunohistochemical analysis was performed. Twelve weeks after the implantation of a P2544-1 hydrogel seeded with GFP-SPC-01 cells to treat an acute injury, the implanted cells survived and maintained a neural phenotype with positive immunostaining for nestin (Fig. 7 C, D), βIII-tubulin (Fig. 7E) and GFAP (Fig.7 F). Some of the grafted SPC-01 cells showed an astrocyte-like phenotype (Fig. 7 G). The major portion of the implanted cells migrated up to 4mm away from the hydrogel-tissue border (Fig. 7 A, B), while a low number of cells persisted in the hydrogel material. The hydrogel's intrinsic environment was shown to be partially penetrable by connective tissue elements.

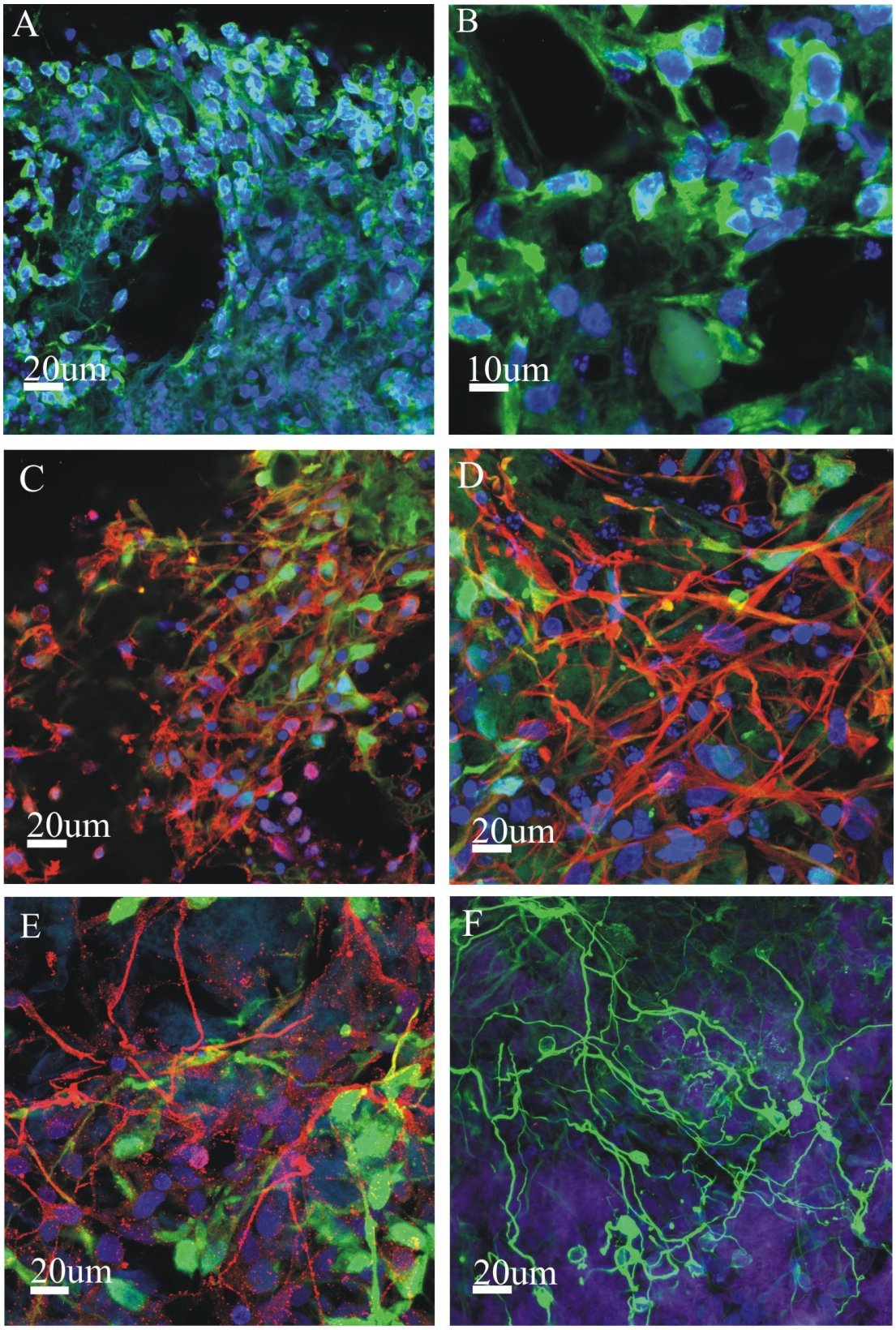


Fig. 6. Twenty-eight days after GFP-human fetal neural stem cell (SPC-01) seeding on a P2544-1 hydrogel. The HEMA hydrogel containing serotonin agonists was filled to confluence by human fetal neural stem cells. The SPC-01_GFP3 cells showed positivity for neuroectodermal markers: neurofilaments 70 (A) - higher magnification (B); neuronal- cell adhesive molecule (C); nestin (D); synaptophysin (E); βIII-tubulin (F).

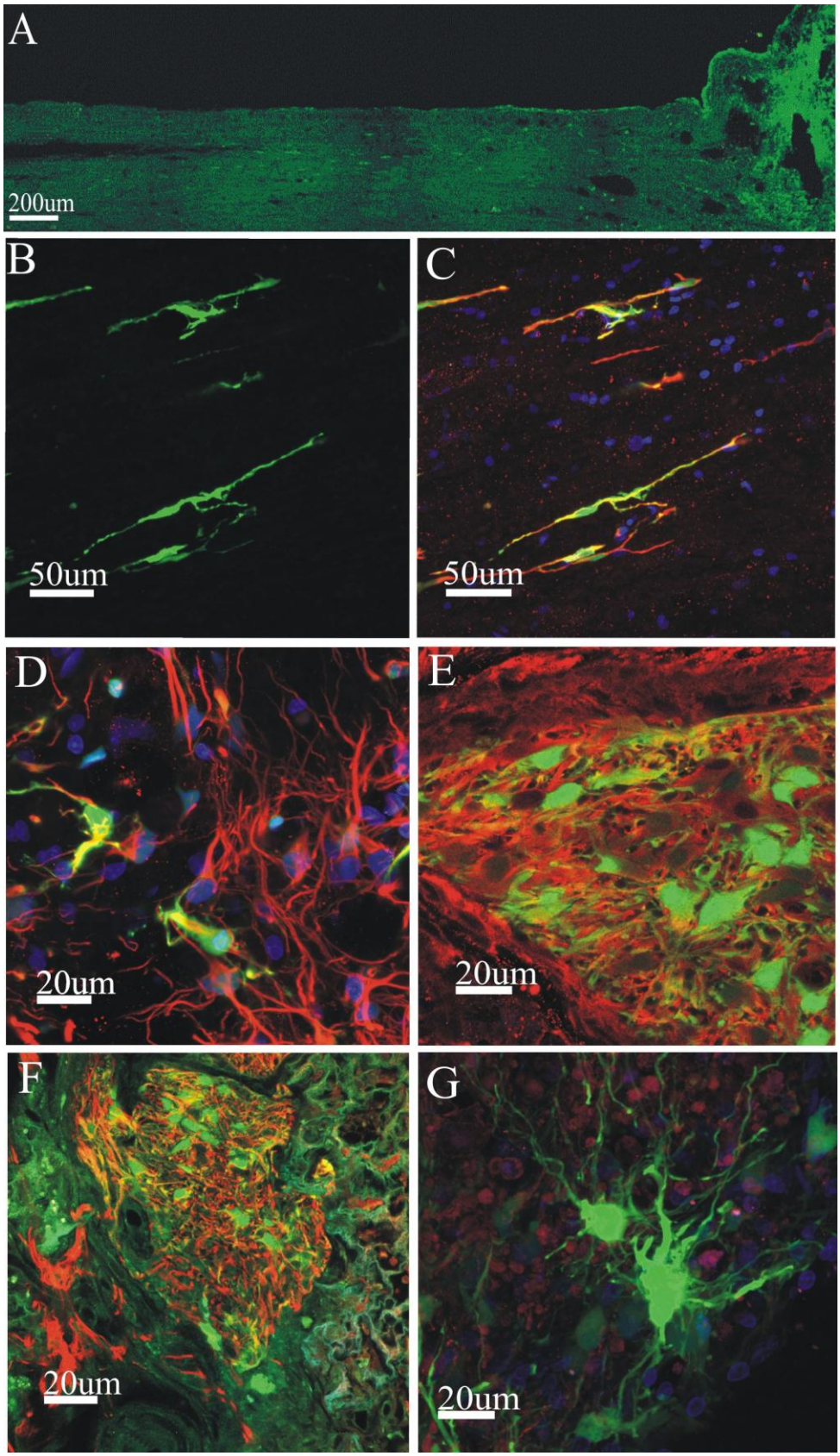


Fig.7. Three months after the acute implantation of human fetal neural stem cells seeded on a P2544-1 hydrogel into a spinal cord hemisection cavity. SPC-01_GFP3 cells migrated up to 4 mm cranially from the implanted hydrogel (A; detailed view B) and remained immunopositive for neural phenotype markers: nestin (C, D); βIII-tubulin (E); GFAP (F). Implanted GFP-SPC-01 cells also show an astrocyte- like phenotype (G).

5.2. Behavioural evaluation

5.2.1. BBB test

The BBB locomotor score test was used to evaluate functional locomotor improvement after SCI. We tested both the treated and the control group of animals starting one week after injury. The H0 hypothesis for the equal variance of data gained from each testing procedure was not dismissed at the 5% reliability level. A significant difference between the treated and control groups was found the first week after injury (t-test, $p < 0.02$). Animals of the treated group achieved an initial mean score of 10.36 ±1.38 (standard error of the mean) on the BBB rating scale compared to 8 ±1.56 for animals from the control group. These differences maintained the same trend for the second and third weeks after injury but with no statistically significant difference (Fig.8). Differences between the groups were more clearly visible on the left hind limb (Fig.9A, B). From the fourth week after injury to the end of the study, no statistically significant differences between the groups were observed. The final mean scores that the animals reached were 12.25 ±0.90 for the treated animals compared to 12.61 ±1.58 for the control group.

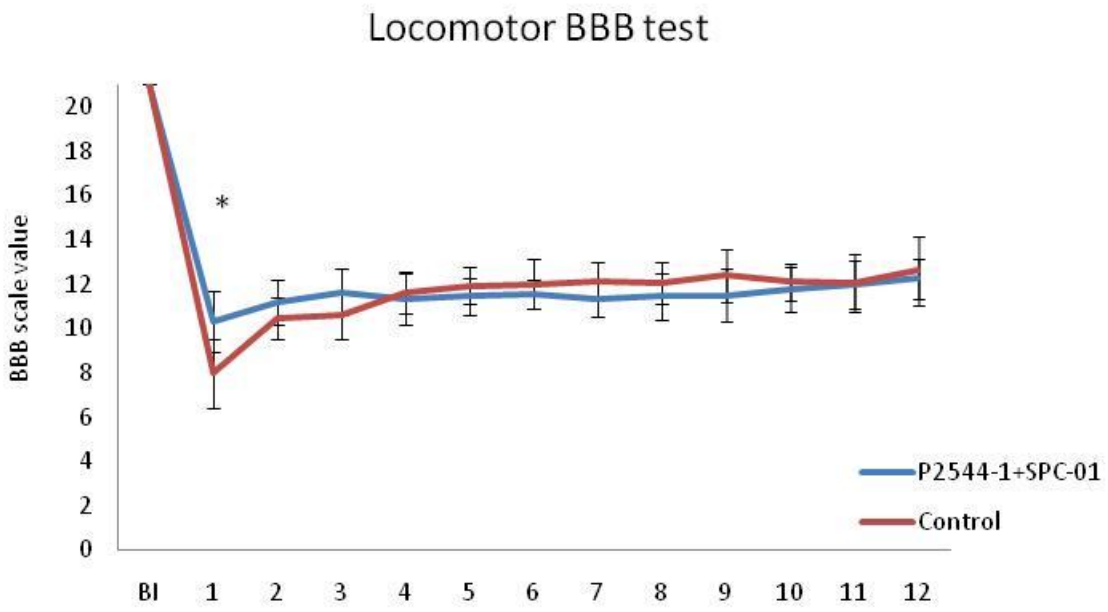
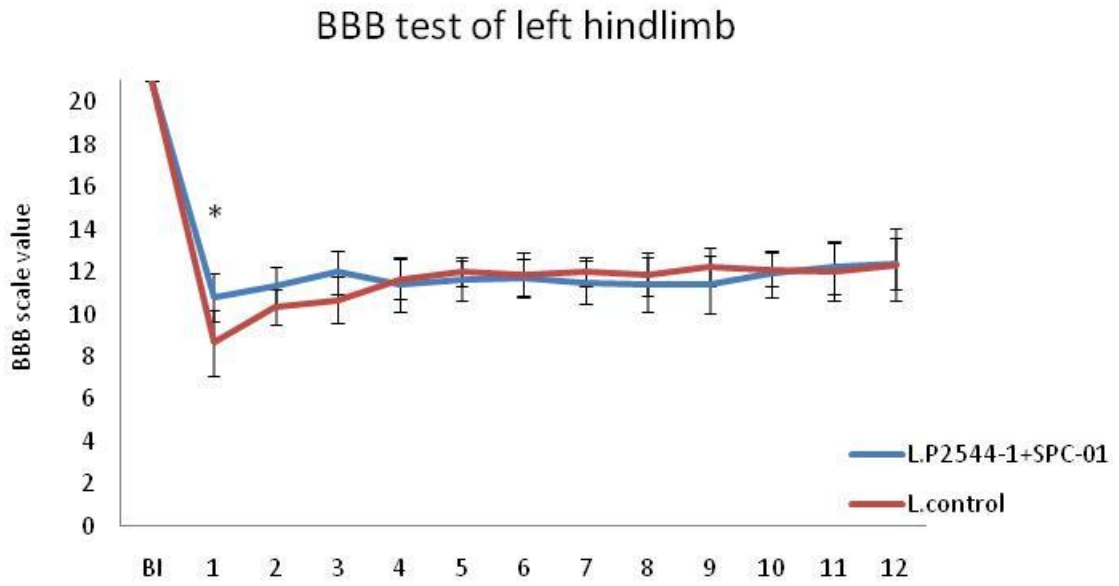


Fig.8. Locomotor score of treated and control animals measured using the BBB rating scale (Tab. 2) once a week (1- 12), starting in week before injury (BI). Arithmetical mean of the scores from both hindlimbs.

A



B

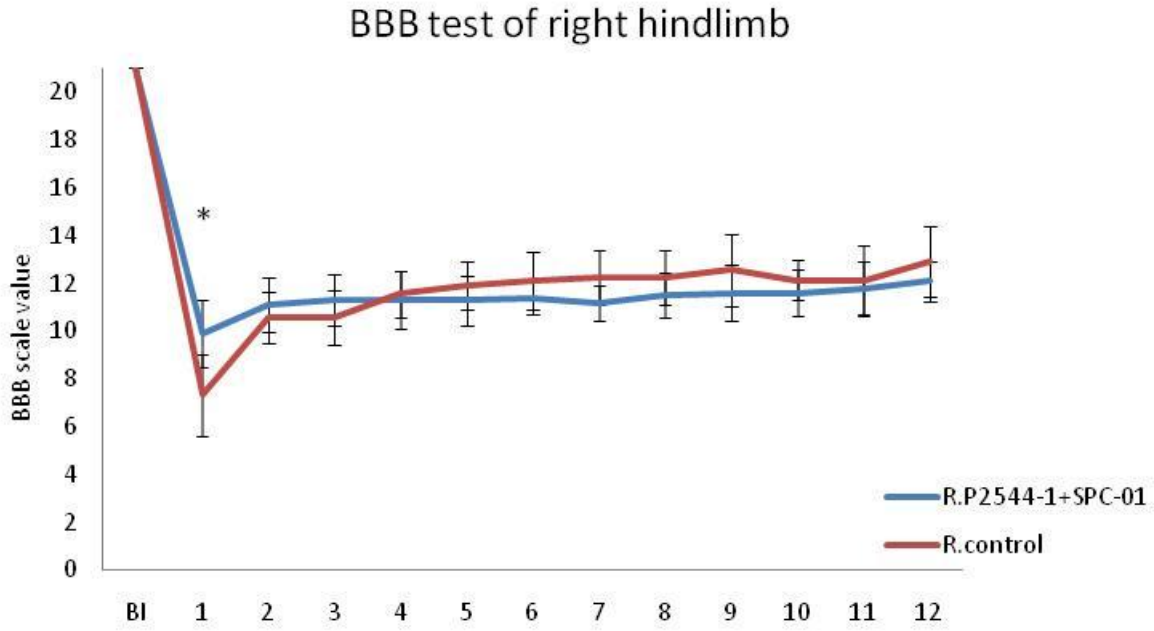
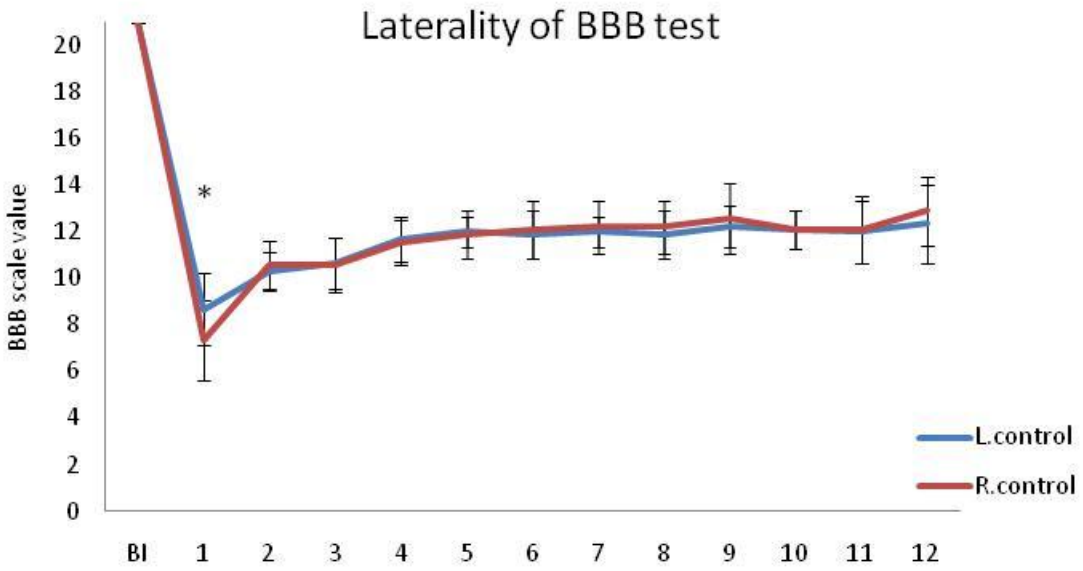


Fig.9. Locomotor score of treated and control animals measured using the BBB rating scale (Tab. 2) once a week (1- 12), starting in week before injury (BI). Arithmetical mean of the scores from both hindlimbs. Detail of the left (A) and right hindlimb (B).

The lateralisation effect of the hemisection model of SCI was observed only at the beginning of the experiment (Fig.10 A, B). The treated group showed a statistically significant difference compared to the control group (1^{st} w., $p< 0.01$; 3^{td} w., $p=0.01$; 11^{th} w., $p< 0.04$ VS 1^{st} w., $p= 0.01$) at three time points. The treated group showed a short term

protection of the injured hindlimb compared to the control group. The effect of treatment on locomotor improvement was limited.

A



B

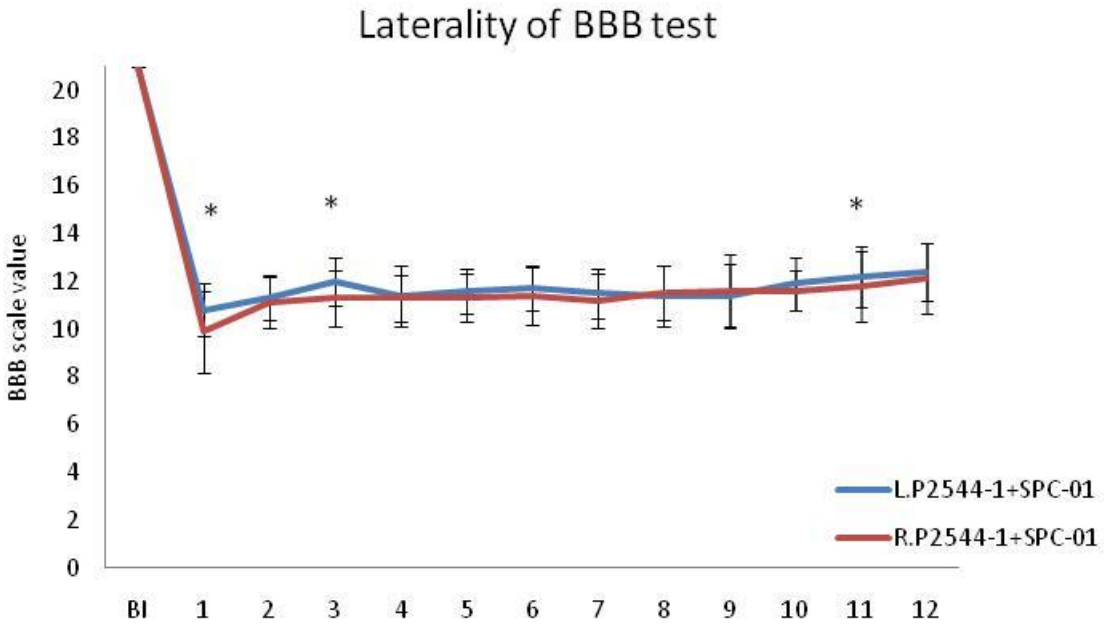


Fig.10. Locomotor score of treated and control animals measured using the BBB rating scale (Tab. 2) once a week (1- 12), starting in week before injury (BI). Arithmetical mean of the scores from both hindlimbs. Laterality of the control (A) and treated group (B).

5.2.2. Plantar test

Thermal sensory system recovery was evaluated using the plantar/ hot plate test. We tested both the treated and control animals, starting in the week before injury (BI, healthy animals) and then week after injury (1), with the pause in week of injury. The H0 hypothesis for the equal variance of data obtained from each testing procedure was not dismissed at the 5% reliability level. The time needed to respond to an infrared light (IR) stimulus measured in both groups before injury did not show any difference between the control (10.50 ±1.24 sec.) and treated (10.55 ±1.71 sec) groups (BO. P= 0.94) and could serve as the normal response time of a healthy animal. No significant differences in the response time (means for both hindlimbs) were observed between the groups during the course of the experiment (Fig.11). The treated animals maintained their response time (1st w 9.60 ±1.50 sec.; 2nd w 9.90 ±1.85 sec.) for two weeks after injury close to the value seen pre-injury, in contrast to the control group (1st w 8.71 ±1.80 sec.; 2nd w 8.04 ±1.26 sec.) in which the response time decreased immediately after injury (Fig.12 A, B). The values of the control group after this initial decrease remained stable for the rest of the experiment. On the other hand, the values of the treated group started to decrease after the first 2 weeks, but with irregular peaks. The response time twelve weeks after injury in animals treated with an SPC-01seeded P2544-1 hydrogel decreased to 6.97 ±2.23 seconds, compared to.7.95 ±1.39 seconds in the control animals. Linear regression analysis of the plantar test results in the treated group showed a steeper trend compared to the control group. The differences were more clearly visible in the results of the left hindlimb (Fig.13 A, B).

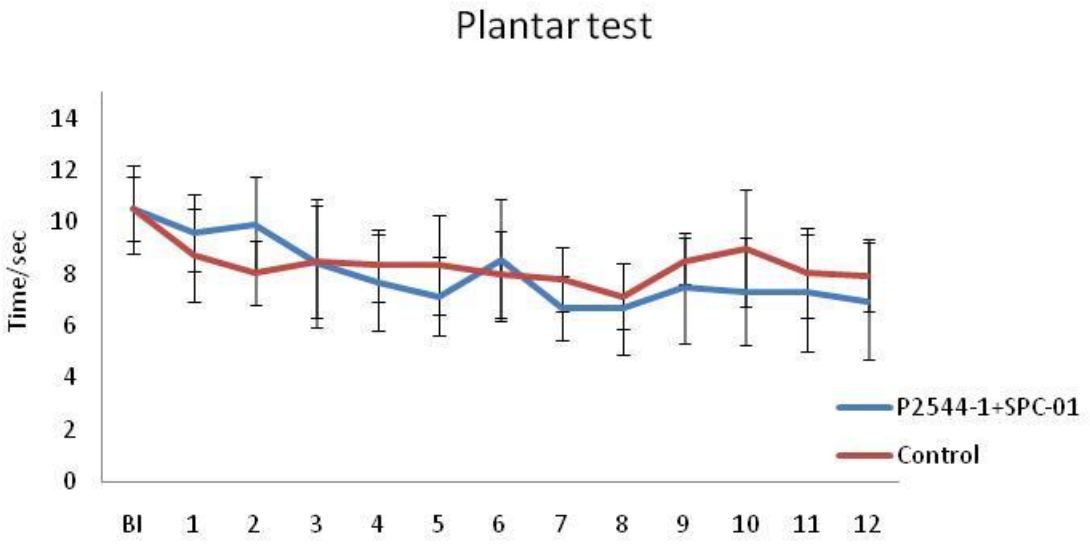
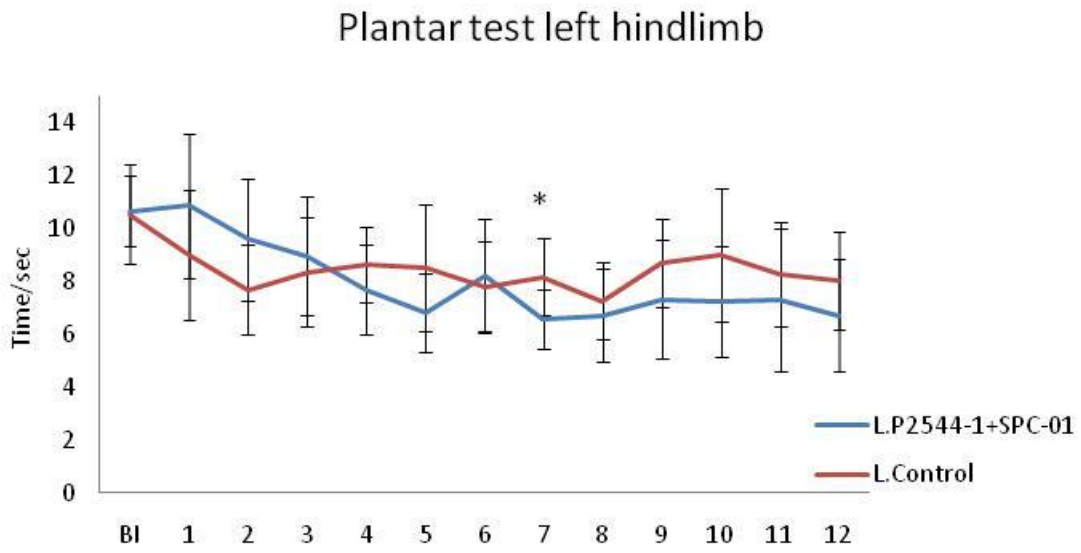


Fig.11. Latency in reacting to a thermal nociceptive IR stimulus measured on the hind limbs for twelve weeks after SCI (1-12) and before injury (BI).

A



B

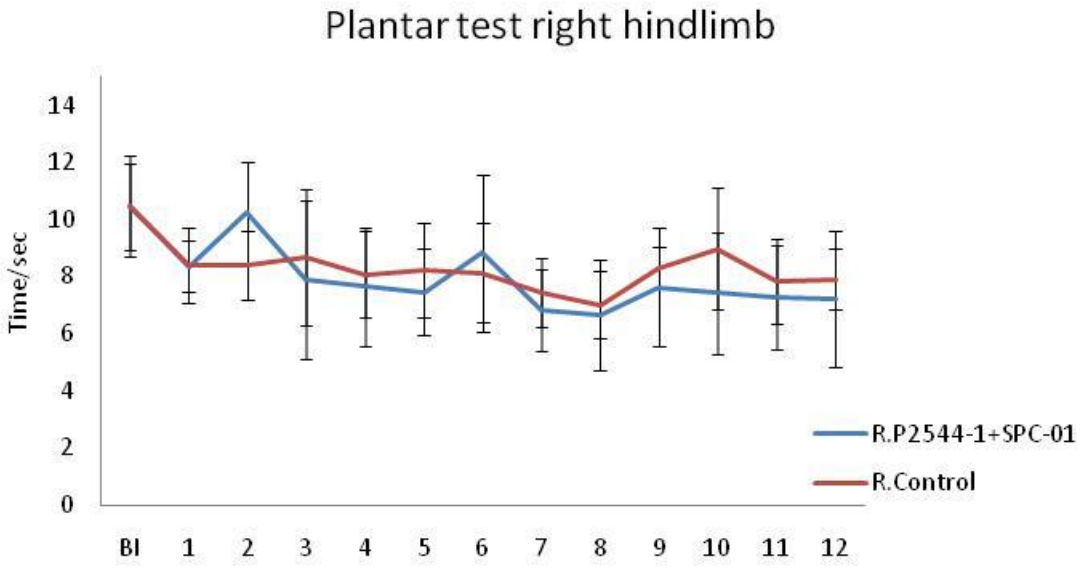
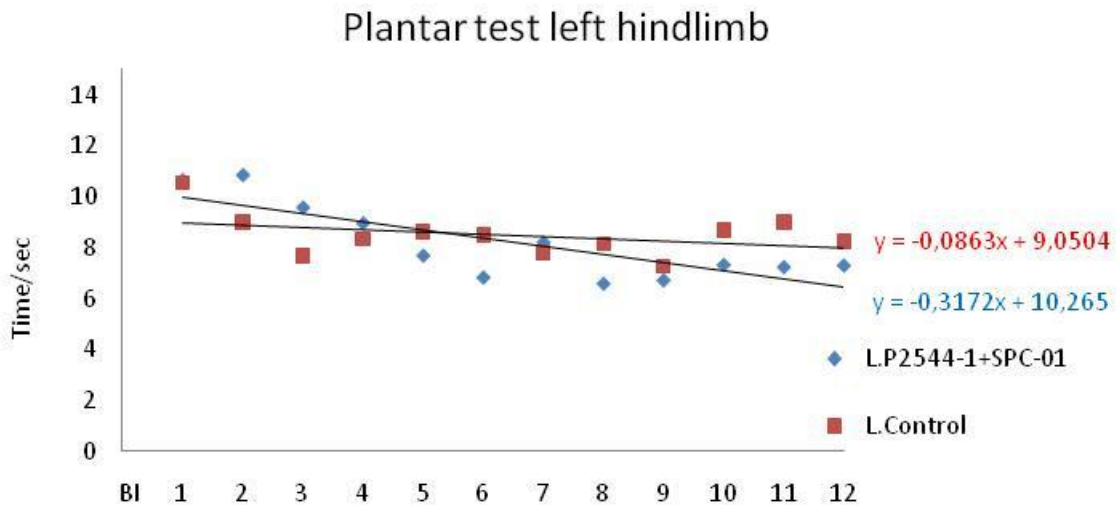


Fig.12. Latency in reacting to a thermal nociceptive IR stimulus measured on the hind limbs for twelve weeks after SCI (1-12) and before injury (BI). Details of the left (A) and right (B) hind limb response times.

A



B

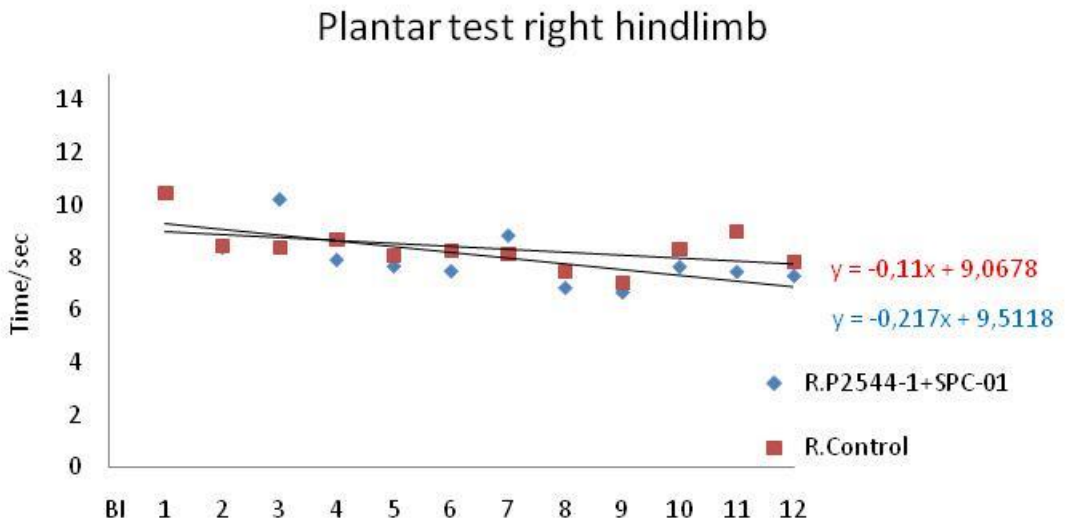
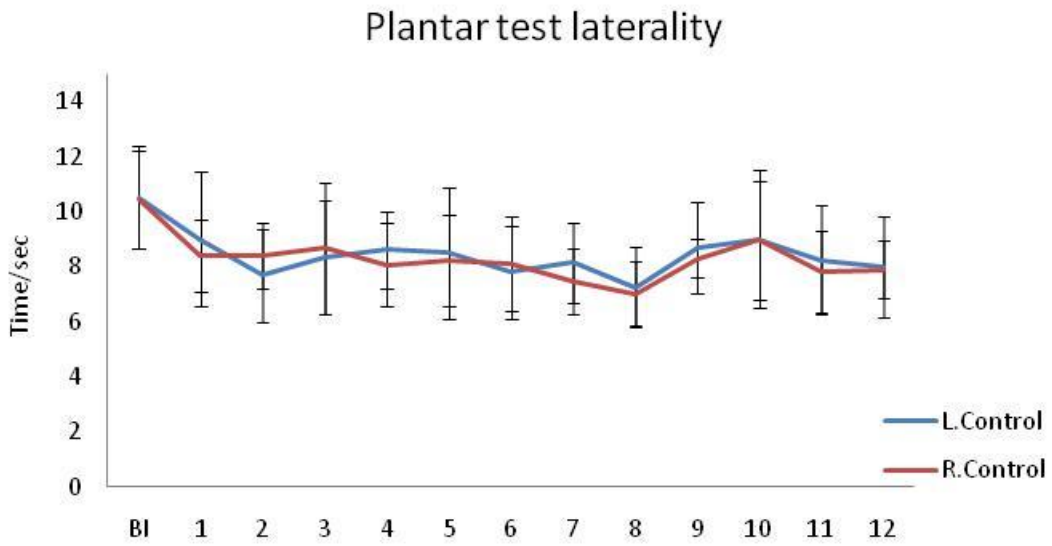


Fig.13. Latency in reacting to a thermal nociceptive IR stimulus measured on the hind limbs for twelve weeks after SCI (1-12) and before injury (BI). Details of the left (A) and right (B) hind limb response times.

A lateralisation effect of the hemisection model was not observed (Fig.14 A, B). The treated group only once showed a statistically significant difference at the 5% reliability level (3^{td} W., $p > 0.04$). The effect of treatment on sensory improvement was limited, and only the trend in the linear regression of the treated group suggests a possible further effect at the chronic stage.

A



B

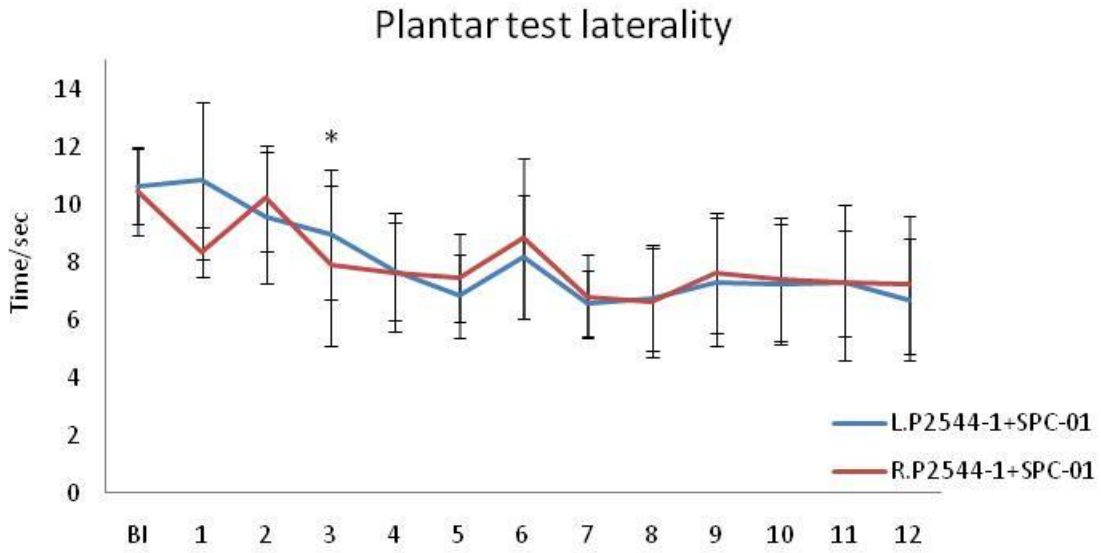


Fig.14. Latency in reacting to a thermal nociceptive IR impulse measured on the hind limbs for twelve weeks after SCI (1-12) and before injury (BI). The lateralisation effect of the injury on thermal nociception observed in animals of the control (A) and treated (B) groups.

5.3. Effect of seeding SPC-01 cells into P2544-1 hydrogels on the injured spine

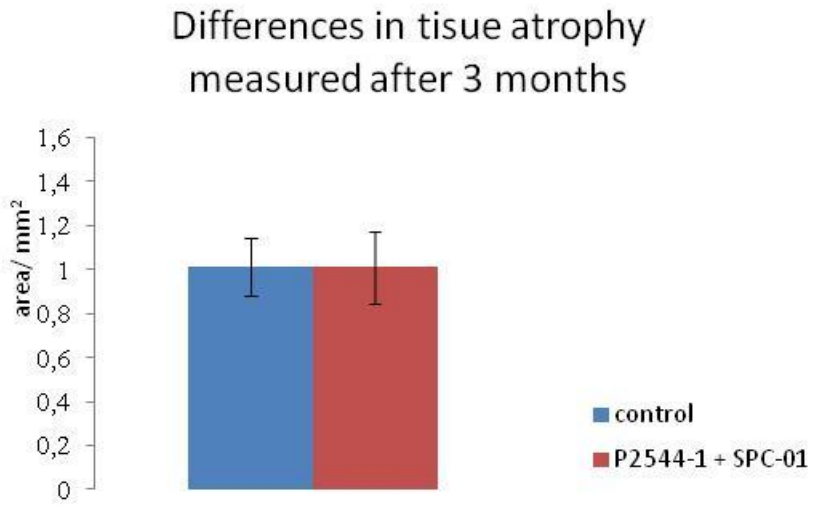
5.3.1. Tissue atrophy

For evaluating the effect of treatment on protecting against tissue atrophy, software measuring the surface area of spinal slices was used. On hematoxylin-eosin stained slices, a difference in spinal tissue atrophy between the control group and the GFP- SPC-01-seeded P2544-1 hydrogel group was not observed (Fig.11.A). In the spines of the animals from the control group, only a small difference in the extent of cavity formation and tissue atrophy was observed compared to the treated group (Fig. 10 A, B).

A significant difference in the atrophy of the spinal cord tissue between the treated and the control group was not observed (F-Test; $p= 0.46$). The extent of the preserved spinal tissue was not significantly higher in the treated group (T-Test; $p= 0.88$). The treated group showed only a 1% larger area of preserved tissue than the control group. The spinal cords of the treated animals showed a significant difference in the amount of preserved tissue one month after injury compared to three months after injury (F-Test; $p= 0.66$; T-Test; $p< 0.01$) (Fig.11.B). These results correspond to the BBB results.

Fig.15. Spinal tissue atrophy visualised three months after a spinal cord hemisection in control animals (A) and treated animals (B). Haematoxylin-eosin staining.

A



B

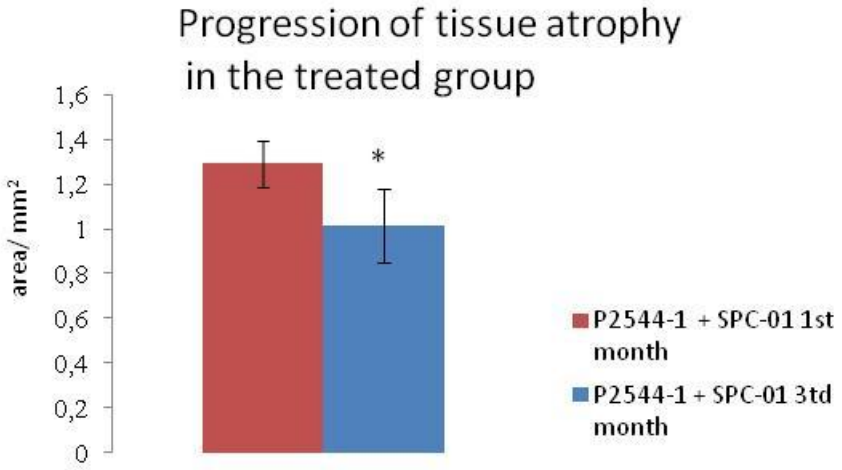


Fig.16. Differences in tissue atrophy measured on HE slices between spinal cords from the control group and from the treated animals (A). Progression of tissue atrophy in the treated group (B).

5.3.2. Astrogliosis

To estimate the effect of treatment on astrogliosis in the surrounding tissue, software measuring the area of positive fluorescence was used. On GFAP-Cy3 stained spinal tissue, a decrease in the extent of astrocyte marker positivity (GFAP) was observed in the treated group compared to the control animals (Fig.17). The area of GFAP-Cy3-positive staining was reduced by 60% in the treated animals Spinal slices from the control and treated groups had an unequal variability of the measured data (F-Test; $p< 0.01$). However, after applying the t-test to the unequal variability data, a statistically significant difference between the control and treated groups was found (T-Test; $p< 0.01$) (Fig.18). The hydrogel was partially penetrated with GFAP-positive cells (Fig.17 A).

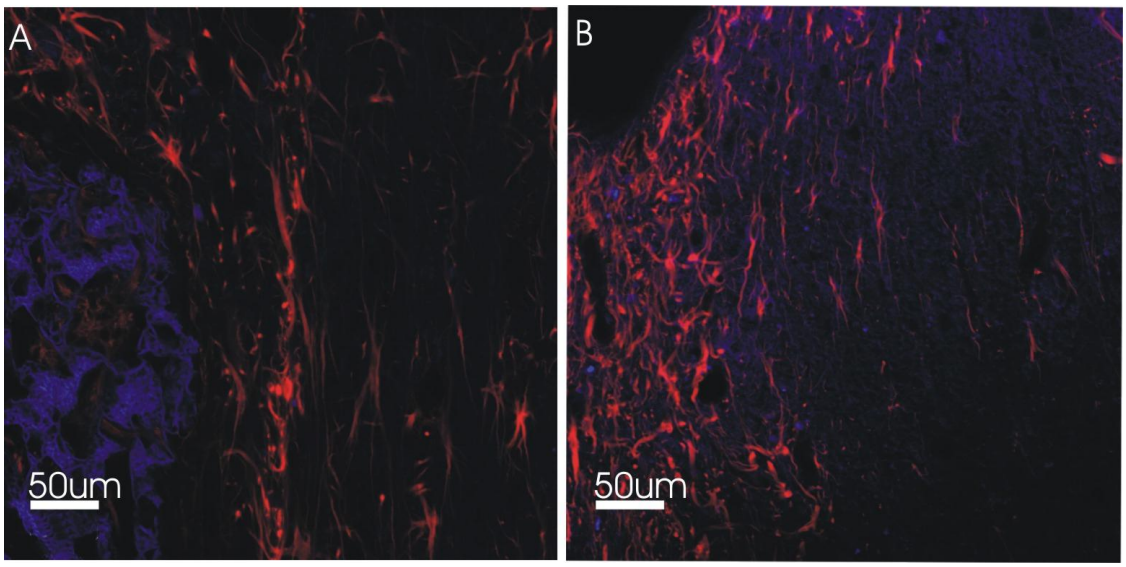


Fig.17. GFAP positivity at the lesion border measured in the treated group (A) and control animals. (B).

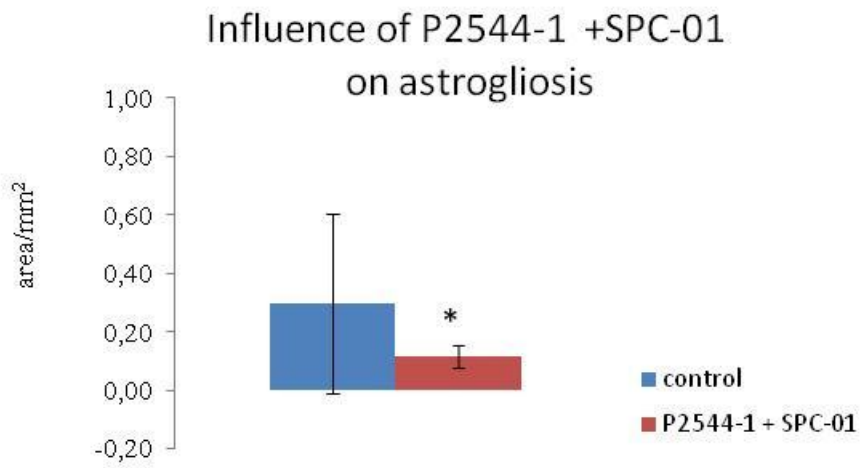


Fig.18. Decrease in astrogliosis after the implantation of a hydrogel seeded with hfNSCs in a hemisection model of SCI.

5.3.3. Prevention of axonal degeneration and axonal sprouting

We were not able to evaluate the degree of axonal degeneration and axonal sprouting in the two groups of animals. However, images of the treated group showed only partial penetration of the border of the hydrogel material (Fig.19). Modification of the analysis method would be appropriate for further analysis.

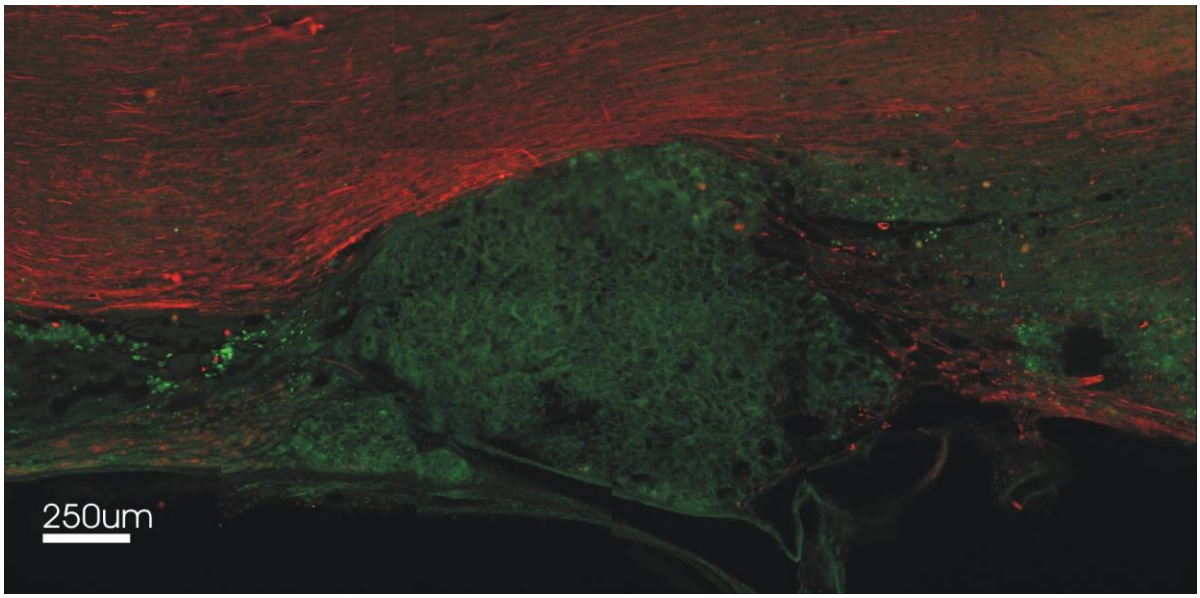


Fig.19. Axonal penetration of P2544-1 hydrogel in hemisection model of SCI visible 3 months after injury.

5.3.4. Revascularisation of the lesion environment and hydrogel penetration

Using a method for quantifying the revascularisation of the lesion, we were unable to find any statistically significant difference between the groups. However, images of the treated group showed marked penetration of the surrounding tissue and partial penetration of the hydrogel (Fig.20). Modification of the analysis method would be appropriate for further analysis.

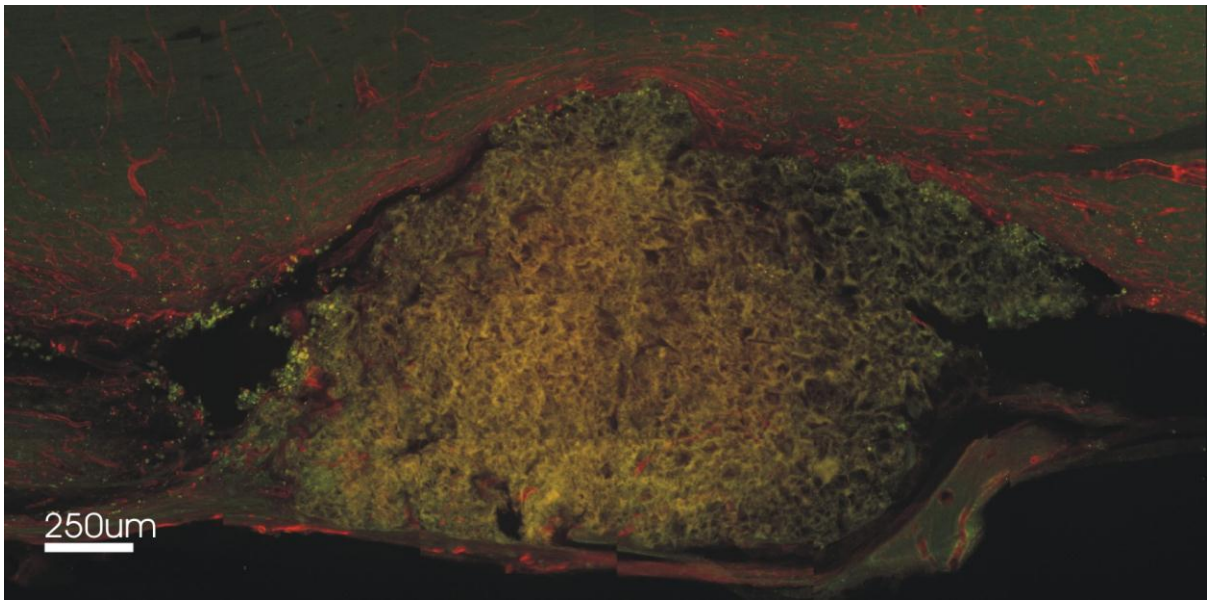


Fig.20. Vascular penetration of a P2544-1 hydrogel in a hemisection model of SCI 3 months after injury.

5.4. Animals

We observed differences in the survival of the animals between the two groups. Of 31 male Whistar rats, only 19 animals survived to the end of the experiment. The mortality was unequal between the control and treatment groups. Whereas in the treatment group only 1 of 11 rats) died, in the control group only 9 of the 20 rats survived to the end of the experiment; four of them died immediately after surgery. Differences in the survival of the animals between the control and the treated groups disappeared immediately after the installation of a system of internally ventilated cages.

6. Discussion

In my Master of Science research, the SPC-01 line of hfNSCs together with P2544-1 hydrogel was used for the treatment of SCI in a rat hemisection model of SCI. No other works have been published yet on the effect of this combination or on the *in vivo* effect of each particular type of treatment. P2544-1 hydrogel was chosen based on our previous *in vitro* study, in which we examined the influence of different concentrations of serotonin agonist molecules covalently bound to HEMA hydrogel on the growth, proliferation and differentiation of NSCs (data not published). The SPC-01 cells were characterised by SSEA-1, NCAM, βIII-tubulin, nestin and CD271 positivity. The main influence on the results of this work was the model of lateral hemisection. The model of lateral hemisection is able to show the penetration of tissue elements into the implanted material and the microstructural effect on the surrounding tissue more clearly, but due to the strong lateral sprouting of the persisting and newly forming axons, any functional effect must be very strong to be detectable at the level of statistically significant differences.

6.1. SPC-01 cells

The reason why we chose hfNSCs as a cellular graft in an animal model is the possibility of its suitability for future human trials.

The SPC-01 line of hfNSCs is one of the neural lineages derived by modifying the c-mycER^{tam} gene. Compared to Pollock's experiments with a human neural stem cell line (CTX0E03), in which the NSCs were prepared for chronic stroke treatment and for clinical trial, the SPC-01 line of hfNSCs derived from 8-week-old human fetal spinal cord is at the beginning of preclinical experiments.

The differentiation of SPC-01 cells under *in vitro* conditions showed P2544-1 hydrogel to be a suitable environment for hfNSC growth. The SPC-01 cell line in the presence of 4OHT factor in the culture medium maintains its neuroectodermal phenotype and proliferates without further differentiation. However, under conditions in which cell culture growth exceeds 80% confluence, the cells are able to differentiate into a neural phenotype even in the presence of 4OHT. Because the cultured cells were not influenced by growth factors or morphogens, the process of differentiation was attenuated by the cell density. hfNSCs derived from the spinal cord are multipotent cells, and their differentiation potential was directed towards a neural phenotype. After implanting the hydrogel material with

cultured cells into the aggressive injury environment, the P2544-1 hydrogel maintained its attractiveness for hfNSCs, but environmental changes and the natural migratory potential of SPC-01-derived NSCs were stronger. After migration, the trophic effect of the cells was divided between the injured and the healthy parts of the spinal tissue. The potential of a serotonin agonist as an attractant proved to be sufficient for new tissue ingrowth, but for anchoring the implanted cells in the CNS, primary AA sequences of extracellular matrix proteins may be attached to the biomaterial (Lieb et al., 2005).

We did not perform absolute counts of the implanted GFP-positive cells, but such cells were highly visible throughout a large area of the injured spinal cord. Those cells that persisted in the hydrogel survived in lower numbers due to the latency of hydrogel vascularisation and only partial innervation. Compared to immortalised NSCs without factor "delinealization" (Lee et al., 2007 A), or other local stem cells, human fetal spinal cord-derived NSCs are in their own niche. The neural phenotype of the implanted cells seeded on the hydrogel observed after three months of *in vitro* culturing and *in vivo* survival and proliferation was not at the stage of terminal differentiation, because the length of the *in vivo* study was shorter than that needed for the endogenous differentiation human NSCs into mature neurons. In order to determine whether the differentiation process of the implanted cells copied the endogenous one, more results from ongoing experiments would be necessary.

6.2. The combined effect of P2544-1 and hfNSCs on damaged spinal tissue

In different studies HEMA hydrogels and NSCs have shown their effectiveness in SCI repair. Thus, four parameters (tissue atrophy, astrogliosis, vascularisation and innervation) were chosen to compare the effect of combined treatment on the morphology of the spinal cord.

In terms of decreasing tissue atrophy, both parts of the treatment process (stem cells and hydrogel implants) have shown their effectiveness in several previous studies. Different types of NSCs have shown the ability to decrease cell apoptosis, produce trophic factors and replenish the damaged white and grey matter (Blesch et al., 2002; Cao et al., 2002 Karimi-Abdolrezaee et al., 2006; Lee et al., 2008; Lu et al., 2003). Hydrogel implants decrease atrophy by their smooth connection to the neural tissue, decrease cavity formation (Hejcl et al., 2008 A; Teng et al., 2002; Woerly et al., 2001) and decrease secondary injury processes including inflammation. Hydrogels have shown that they are able to serve as a bridge (Lesny et al., 2002; Woerly et al., 2001) and to either store or release trophic factors (Brandl et al., 2010). Thus, it is quite interesting that in our experiments utilizing the combined treatment of

SPC-01hfNSCs and P2544-1 hydrogel, we observed no effect on tissue atrophy. However, this negative observation is in agreement with our behavioural testing results, which showed no statistically significant positive effects after the first week post-injury. An effect on cavity formation was not apparent in comparison to the control animals. The results one month after SCI showed only a short-term effect on spinal cord microstructure.

The differences in the size of the GFAP-positive area between the treated group and the controls are possibly caused by a combination of biological factors. In terms of biological relevance, the influence of possible differences between the groups played a counterproductive role. On one hand the endogenous potential of the spinal environment pushed the differentiation of the NSCs, in part, towards an astrocyte-like GFAP-positive phenotype. On the other hand, the potential of the hydrogel to suppress astrogliosis played a role. Since GFAP was used as a marker of astrocytes for the analysis, and since GFAP expression was present on a portion of the implanted cells, the reduction of astrogliosis in the endogenous tissue was even greater than the data indicate. In the experiments of Woerly and his colleagues, the implantation of a HPMA hydrogel resulted in a statistically significant decrease of glial scarring in the lesion (Duconseille et al., 1998; Woerly et al., 2004). However, experiments using different types of modified HEMA hydrogel have not yet shown such ability. A hydrogel that releases ChABC into the astrocytic scar surrounding the lesion is an exception (Hyatt et al., 2010). The suppression of astrogliosis plays a role in renewing the connectivity between the spinal cord stumps. This valuable positive effect of HEMA hydrogels containing serotonin agonists is a step forward towards artificial tissue engineering

Blood vessels and endogenous axons penetrated the hydrogel material only partially. Without the possibility of objective quantification, it can only be said that the penetration into the material was not strong or fast enough to prolong the effect of the non-migrating part of the cell population within the implanted material. The fact that the hydrogel material was completely filled with cells *in vitro* points to the problem of early *in vivo* revascularisation and neural penetration. Whereas the *in vitro* environment allows for the simple diffusion of medium and metabolites, the *in vivo* situation decreased diffusion and, together with unidirectional pores, led to lower cell density. The penetration of blood vessels proved to be more promising compared to the penetration of endogenous axons and other tissue elements, such as astrocytes. These differences can be explained by the fact that the macroporous network on one hand offers space for penetration, but on the other hand offers no strong, localized gradient necessary for attracting axons. In this way the covalently bound serotonin agonist and the pore network characteristic of the P2544-1 hydrogel do not represent effective

attractants for a long distance gradient. The effect of hydrogel density and porosity on tissue penetration is still under observation, but it is crucial for the survival and proliferation of the implanted cells (Moon et al., 2010; Dziubla and Lowman, 2004; Stokols and Tuszynski, 2004; Zawko et al., 2010).

6.3. Effect on functional recovery

There are probably several reasons why the combined treatment of a hemisection model of SCI utilizing P2544-1 hydrogels seeded with SPC-01 hfNSCs resulted in no significant improvement in sensory or motor recovery. First, it is important to point out that both parts of the treatment, hydrogels and NSCs, have shown their potential in the past. At the start of the treatment, the seeded hydrogel kept the uninjured portion of the spinal cord, with its spared axons, separated from the injured tissue, with the result that the undamaged tissue was less affected by secondary injury processes. Nevertheless, after a few weeks no differences in locomotor recovery were observed between the two groups of animals. The data from the plantar test were markedly affected by variability within the groups; to decrease this variability, more animals would need to be included in the study. One of the ways in which potential differences between the treated/ control groups and between the left/right hindlimbs could be decreased is lateral sprouting, typical of incomplete, lateralised hemisection injuries. Compared to the more complex closed model of a compression injury, in the hemisection model the positive effect of SPC-01 hfNSC-seeded P2544-1 hydrogel on sensorimotor recovery may not be so obvious.

The second reason for not finding any significant difference could be the fact that the hydrogel did not contain oriented pores throughout the entire hydrogel volume and thus did not provide directed guidance to ingrowing axons and other tissue elements. The hydrogel supported SPC-01 growth, as observed in the vitro part of the experiment, but the penetration of newly formed or regenerated axons through the hydrogel should be directed by a gradient of signal molecules, attracting axonal growth in order to reconnect the spinal cord stumps. In addition, the channel network should be easily penetrable by the vascular system, because a time delay in vascular penetration adversely affects the survival of cells in the centre of the hydrogel (Dziubla and Lowman, 2004).

The third reason could be the unrealized potential of hfNSCs to differentiate into mature neurons and glial cells as a result of the limited time span of our experiments. In the process of regeneration after injury, trophic factors produced by stem cells and increased neuronal plasticity both play a role, but the duration of our study did not allow the terminal

differentiation of the implanted cells for functional replacement of the damaged circuits. The significant effect of treatment on astrogliosis had no influence on the results of the testing procedures used to evaluate recovery.

Because of the low sensitivity of our battery of tests, a more extensive testing procedure could detect specific differences. Supplementing the behavioural evaluation, for example, with the tactile allodynia test (Von Frey filament test) or electrophysiology (EMG) could bring additional information and demonstrate a possible positive effect of the treatment on functional recovery.

6.4. Animals

The reasons why the animals in the treatment group had a statistically significant lower mortality compared to the control group are complex, and without microbiological examination, they can be only guessed at. Both groups were immunosuppressed with cyclosporine-A, selectively acting on the T-cell immune response. The secondary injury processes were co-treated with MPSS. Animals with decreased immunity, which could be further affected by the side effects of MPSS administration, were certainly more at risk from possible infection. After changing the animals' home cages to an internally ventilated cage system, the differences in mortality disappeared. The lower mortality in the treated group can be explained by the combined preventive effect of hfNSCs and HEMA hydrogel with serotonin agonists. This effect could be caused either by the production of neurotrophic factors such as NGF, BDNF, GDNF or NT3 by the hfNSCs, possibly similar as in other neural precursor cells (Rossi and Keirstead, 2009), or by a decrease in cell apoptosis and tissue atrophy after SCI by modulating the inflammatory response, possibly a similar effect to that observed with NSCs derived from the fetal brain (Lee et al., 2008). However, these effects of SPC-01 hfNSCs have not yet been confirmed.

7. Conclusion

The combination of a hydroxy ethyl methacrylate hydrogel containing serotonin agonists seeded with hfNSCs showed limited effectiveness in promoting locomotor and sensory recovery following spinal cord injury. The properties of the hydrogel are very suitable for the growth of hfNSCs, but after implantation into the injured spine, the P2544-1 hydrogels were not fully penetrated by blood vessels, axons, etc. and did not strongly influence the environment surrounding the lesion. This type of hydrogel displayed considerable promise *in vivo* due to its ability to facilitate astrocyte scar remodelling and short-term stabilisation of the lesion environment, but the combination of hydrogel and hfNSCs was not able to fulfil its initial potential for effective treatment. The effect of this combination (P2544-1 hydrogel+ human fetal neural stem cells) on behavioural recovery could be better demonstrated by using a balloon compression model of closed spinal cord injury.

SPC-01 hfNSCs, on the other hand, proved to be a promising cell type for cell replacement therapy thanks to their ability to survive at the site of transplantation, their migratory potential and their ability to maintain a neural phenotype despite an aggressive environment following injury. For proper evaluation of their long term effect, a model of chronic injury would be necessary, particularly in view of the time needed for their terminal differentiation. Thus, further investigation is necessary to fully reveal the therapeutic potential of SPC-01 cells.

Ongoing and future approaches to spinal cord injury treatment should lead to more specific biocompatible materials that encourage full penetration by endogenous tissue elements as well as to a combination of surface modifications to enhance reconstruction of the lesion environment. Genetic modifications of the implanted cells that would influence their differentiation, improve their survival and facilitate the release of trophic factors will play a major role in future experiments.

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